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(54) Title: MODULATING IL-13 ACTIVITY USING MUTATED IL-13 MOLECULES THAT ARE ANTAGONISTS OR AGONISTS OF IL-13

(57) Abstract: The present invention provides antagonists and agonists of IL-13 activity. The antagonists comprise a mutation in which the glutamic acid at position 13 of the IL-13 molecule is changed to a neutral or a positively charged molecule. The antagonists can be used to reduce or end symptoms in conditions, such as asthma, allergic rhinitis, atopic dermatitis, and hepatic fibrosis in schistosomiasis, in which IL-13 is an initiator, mediator, or enhancer of the abnormal state. Additionally, the antagonists can be used to slow the growth of cells of cancers for which IL-13 is an autocrine growth factor. Such cancers include renal cell carcinoma, Kaposi's sarcoma, and Hodgkin's disease. The agonists comprise mutated IL-13s in which one or more of the residues at positions 112, 110, 109, 92, 69, or 66 are mutated to a neutrally charged residue, or one with a charge opposite to the charge of the residue found at that position in native IL-13, provided that the residue at position 13 of the molecule is not negatively charged. The agonists can be used as more potent agents to provoke an effect provided by IL-13. In particular, the agonists can be used as reagents in the maturation of monocytes into dendritic cells, or to pretreat bone marrow stem cell donors to reduce graft versus host disease in the recipient of the stem cells. Finally, the invention provides IL-13 receptor binding molecules with affinity for the IL-13 receptor at least about 3 times greater than that exhibited by wild-type IL-13. Also provided are methods and compositions for specifically delivering an effector molecule to a tumor cell by chimeric molecules comprising the effector molecule and an IL-13 receptor binding molecule, and pharmaceutical compositions comprising such chimeric molecules.

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MODULATING IL-13 ACTIVITY USING MUTATED IL-13 MOLECULES THAT ARE ANTAGONISTS OR AGONISTS OF IL-13

RELATED APPLICATIONS

This is a continuation-in-part of U.S. Provisional Application Serial No. 60/165,236, filed November 11, 1999, the contents of which are incorporated by reference.

STATEMENT REGARDING FEDERALLY SUPPORTED RESEARCH

Not applicable.

FIELD OF THE INVENTION

This invention relates to mutated forms of IL-13 which have higher binding affinity for the IL-13 receptor than does wild-type IL-13. The invention further relates to uses and medicaments of various of these mutants which are antagonists or agonists, respectively, of IL-13 mediated activity. The invention further relates to targeted delivery of agents to cells overexpressing receptors to IL-13 by using certain of these IL-13 mutants as targeting moieties.

BACKGROUND OF THE INVENTION

IL-13 is a pleiotropic cytokine which plays a major role in immune response and inflammation. (Minty, A. *et al.*, *Nature* 362:248-250 (1993); Michel, G. *et al.*, *J. Invest. Derm.* 103:433-433 (1994); McKenzie, A.N.J. *et al.*, *Proc Natl Acad Sci (USA)* 90:3735-3739 (1993). It can inhibit production of proinflammatory cytokines IL-1, IL-6, TNF-alpha and downregulate the expression of CD14 on monocytes. (Cosentino, G. *et al.*, *J. Immunol.* 155:3145-3151 (1995)). CD14 is a LPS receptor which is important for monocyte functions. (Lauer, R.P. *et al*, *J. Immunol.* 145:1390 (1990); Wright, S.D. *et al*, *Science* 249:1431 (1990)). IL-13 also plays a major role in B cells. It can upregulate CD23, CD72, MHC class II, surface IgM on B cells; drive IgE class switch and induce

production of immunoglobulins by B cells. (Punnonen, J. *et al.*, *Proc Natl Acad Sci (USA)* 90:3730-3734 (1993)).

Recent studies have demonstrated IL-13 plays a prominent role in atopic dermatitis (Akdis, M. et al., *J. Immunol.* **159**:4611-4619 (1997); Katagiri, K. et al., *Clin. Exp. Immunol.* **108**:289-294 (1997)), allergic rhinitis (Pawankar, R. U. et al., *Am. J. Respir. Crit. Care Med.* **152**:2059-2067 (1995)) and pulmonary asthma (Wills-Karp, M. et al., *Science* **282**:2258-2261 (1998); Grunig, G. et al., *Science* **282**:2261-2263 (1998)). Targeted pulmonary expression of IL-13 can cause severe pulmonary pathology including mononuclear and eosinophilic inflammatory response, mucus cell metaplasia, airway fibrosis and obstruction, among other things (Zhou, Z. et al., *J. Clin. Invest.* **103**:779-788 (1999)), as well as hepatic fibrosis induced by Schistosomiasis (Chiaramonte, M. G. et al., *J Clin Investig* **104**:777 (1999)), and susceptibility to Leishmania major infection (Matthews, D. J. et al., *J Immunol* **164**:1458 (2000)). IL-13 also plays a major role in malignancies. It has been shown to be produced by various cancer cell types including renal cell carcinoma (Obiri, N. I. et al., *Clin. Cancer Res.* **2**:1743-1749 (1996)). IL-13 has also been shown to be an autocrine growth factor for Hodgkin/Reed-Sternberg tumor cells (Kapp, U. et al., *J. Exp. Med.* **189**:1939-1945 (1999); Fricker, J. *Mol. Med. Today* **5**:463-463 (1999)).

The receptors for IL-13 have been identified on a variety of normal and malignant cell types (Vita, N. et al., *J. Biol. Chem.* **270**:3512-3517 (1995); Hilton, D. J. et al., *Proc. Natl. Acad. Sci. U.S.A.* **93**:497-501 (1996); Zurawski, S. M. et al., *J. Biol. Chem.* **270**:13869-13878 (1995); Debinski, W. et al., *Clin. Cancer Res.* **1**:1253-1258 (1995); Debinski, W. et al., *J. Biol. Chem.* **270**:16775-16780 (1995); Obiri, N. I. et al., *J. Biol. Chem.* **270**:8797-8804 (1995); Murata, T. et al., *J. Immunol.* **156**:2972-2978 (1996); Puri, R. K. et al., *Blood* **87**:4333-4339 (1996); Debinski, W. et al., *J. Biol. Chem.* **271**:22428-22433 (1996); Aman, M. J. et al., *J. Biol. Chem.* 271:29265-29270 (1996); Murata, T. et al., *Biochem. Biophys. Res. Commun.* **238**:90-94 (1997); Obiri, N. I. et al., *J. Biol. Chem.* **272**:20251-20258 (1997); Husain, S. R. et al., *Clin. Cancer Res.* **3**:151-156 (1997); Murata, T. et al., *Int. J. Cancer* **70**:230-240 (1997); Obiri, N. I. et al., *J. Immunol.* **158**:756-764 (1997); Maini, A. et al., *J. Urol.* **158**:948-953 (1997); Debinski, W. et al., *Int. J. Cancer* **76**:547-551 (1998); Murata, T. et al., *Int. J. Mol. Med.* **1**:551-557 (1998); Debinski, W. et al., *Nat. Biotechnol.* **16**:449-453 (1998); Murata, T. et al., *Blood* **91**:3884-3891 (1998); Joshi, B. H. et al., *Cancer Res.* **60**:1168-1172 (2000)). IL-13 receptors (IL-

13R) are over expressed on human solid cancer cell lines including renal cell carcinoma, AIDS-associated Kaposi's sarcoma, ovarian carcinoma, prostate cancer, and malignant glioma

The function of IL-13 is accomplished through interaction with its plasma membrane receptors. The IL-13 receptor ("IL-13R") complex appears to exist in three different types. In the first type, IL-13 forms a complex with the IL-4 receptor β chain (also known as IL4Rα), IL13R α' (also known as IL13Rα1) and IL-13 R α (also known as IL13Rα2). This type of receptor complex is expressed on limited non-hematopoietic tumor cell lines such as renal cell carcinoma, AIDS-associated Kaposi's sarcoma and glioblastoma multiforme. (Puri, R.K. *et al.*, *Blood* 87:4333-4339 (1996); Husain, S.R. *et al.*, *Clin Cancer Res* 3:151-156 (1997); Debinski, W. *et al.*, *Clin Cancer Res* 1:1253-1258 (1995); Husain, S.R. *et al.*, *Cancer Res* 58:3649-3653 (1998); Husain, S.R. *et al.*, *Nature Med* 5:817-822 (1999)).

In the Type II IL-13 receptor complex, the IL-13Rα chain is not present and IL13 binds to IL-4Rβ and IL-13Rα' chains. The Type II receptor complex is reported to be expressed on some non-hematopoietic malignant cells such as A431, PA-1 and HT-29. In the third type of IL13R, IL4Rβ and IL13Rα' chains may associate with the IL-2R gamma chain (which is known as "gammac"), which is also present in the IL4R, IL7R, IL9R and IL15R systems. (Miyajima, A. *et al.*, *Ann Rev Immunol* 10:295-331 (1992); Kishimoto, T. *et al.*, *Cell* 76:253-262 (1994); Giri, J.G. *et al.*, *Embo Journal* 13:2822-2830 (1994). Type III IL13R is present on hematopoietic cells such as human erythroleukemia cell line TF-1 and healthy human primary monocytes. Although the gammac chain does not interact with IL-13R directly, it modulates IL13R function through downregulation of IL-13Rα, and to some extent α', chains. (Kuznetsov, V.A. et al., *Biophysical Journal* 77:154-172 (1999)).

The IL-4R system, which is related to the IL13R system, also exists in three different types. In type I IL4R, the IL4Rβ chain forms a complex with gammac, in type II receptors, the IL4Rβ chain forms a complex with the IL13Ralpha' chain and, in type III receptors, all three chains are present. (Murata, T. *et al.*, (1997) *supra*). From these studies, it has been concluded that the IL4Rβ and IL13Rα' chains are shared between the IL4R and IL13R systems. (Murata, T. *et al.*, *Intl J Mol Med* 1:551-557

(1998)).

Several studies suggest that blocking the effect of IL-13 by soaking up IL-13 with the α chain of the IL-13 receptor (IL-13R), a protein which binds IL-13, can provide therapeutic benefit in various inflammatory diseases in murine models including bronchial asthma (Wills-Karp, M. et al., *Science* **282**:2258-2261 (1998); Grunig, G. et al., *Science* **282**:2261-2263 (1998)), hepatic fibrosis induced by schistosomiasis (Chiaramonte, M. G. et al., *J. Clin. Invest.* **104**:777-785 (1999)), as well as decrease susceptibility to *Leishmania* major infection (Matthews, D. J. et al., *J. Immunol.* **164**:1458-1462 (2000)). In the past, IL-4 antagonists have been used to block the effect of IL-4 in various murine models of inflammatory diseases (Grunewald, S. M. et al., *J. Biol. Chem.* **272**:1480-1483 (1997); Grunewald, S. M. et al., *J. Invest. Dermatol.* **110**:501-501 (1998); Grunewald, S. M. et al., *J. Immunol.* 160:4004-4009 (1998); Carballido, J. M. et al., *J. Cellular Biochem.*, **114**:114 (1995); Carballido, J. M. et al., *J. Immunol.* **155**:4162-4170 (1995)). At least two inhibitors of IL-4, including an IL-4 antagonist and a soluble IL-4Rβ chain, are in the clinic for the treatment of bronchial asthma (Smith, L. J. *Ann. Intern. Med.* **130**:531-532 (1999); Asthmatics, B. G.-Q. (http://www.bayer.com/webzine/asthma/kstudie_en.html) In). Because IL-4 inhibitors not only block the effect of IL-4 but also the effect of IL-13 through shared receptors on some cell types, it is hypothesized that IL-4 mutant can also block the effect of IL-13 in certain systems (Zurawski, S. M. et al., *EMBO J.* **12**:2663-2670 (1993)). However, since the IL-13Rα chain is not shared with the IL-4R, and since this chain binds IL-13 with stronger affinity than does the IL-4R, it has been predicted that IL-4 mutants will not be very useful in blocking the effect of IL-13 on every cell type (Murata, T. et al., *Blood* **91**:3884-3891 (1998)). To overcome this problem, a soluble extracellular domain of IL-13Rα has been fused to Fc portion of human immunoglobulin and the resulting protein (IL-13Rα/Fc chimera) has been found to block the effect of IL-13 *in vitro* and in many models of inflammatory diseases *in vivo* (Donaldson, D. D. et al., *J. Immunol.* **161**:2317-2324 (1998)). Because IL-13 can signal in the absence of IL-13Rα chain, however, it is not likely that soluble receptor fusion protein might not be able to block the effect of IL-13 in every system.

A variety of human solid tumor cells express elevated levels of IL13 receptors. (Obiri, N.I. *et al.*, *J Clin Invest* 91:88-93 (1993); Obiri, N.I. *et al.*, *Clin Exp Immunol* 95:148-155 (1994); Puri, R. *Cytokines: Interleukins and Their Receptors,* 143-

186 (1995); Debinski, W. *et al.*, *J Biol Chem* 270:16775-16780 (1995)). To target these receptors, a chimeric protein composed of IL13 and a mutated form of *Pseudomonas* exotoxin (PE38) was produced. (Debinski, W. *et al.*, *Clin Cancer Res* 1:1253-1258 (1995); Debinski, W. *et al.*, *J Biol Chem* (1995) *supra*). This cytotoxin is highly cytotoxic to IL13R-positive malignancies. (Debinski, W. et al., *Clin Cancer Res* (1995) *supra*; Debinski, W. *et al.*, *J Biol Chem* (1995) *supra*). The results of some of this work are also embodied in U.S. Patents No. 5,614,191 and 5,919,456, both of which are incorporated herein by reference. Unfortunately, the binding affinity of IL13-PE38 was 10 times lower than native, or "wild-type" IL13. (Puri, R.K. *et al.*, *Blood* 87:4333-4339 (1996))

IL-13 and IL-4 share receptors on normal cells. Debinski *et al.*, Nature Biotechnology 16:449-453 (1998) (hereafter, "Debinski *et al.*, 1998" or "Debinski 1998"). It appears that human IL-13 ("hIL-13" or "hIL13") may possess at least two receptor recognition sites, one that recognizes one of the chains of the IL-4 receptor, and another which recognizes the IL-13 receptor (or "IL-13R"). *Id.* Although the crystal structure of IL-13 is not known, recent work has attempted to draw structural analogies from the IL-4 receptor (or "IL-4R") to the IL-13 receptor. Thus, noting that a mutation changing the glutamic acid at position 9 to lysine in IL-4 impaired binding to the IL-4R, and that the glutamic acid at this position was conserved between IL-4 and IL-13, Debinski *et al.*, 1998 created a mutated IL-13 in which the glutamic acid at position 13 (which corresponds to position 9 of IL-4) was changed to lysine. This molecule, styled hIL13.E13K, was found to have only weak proliferative activity (or mitogenic) activity. When coupled to a modified *Pseudomonas* exotoxin, PE4E, the cytotoxin (hIL13.E13K-PE4E) exhibited decreased cytotoxicity to normal cells but increased cytotoxicity to tumor cells expressing IL-13R when compared to native, or wild-type, IL-13-PE4E. In competition assays against wild-type IL-13, hIL13.E13K was found to bind to U-251MG cells with much greater affinity than did wild type hIL13. Debinski *et al.*, 1998. The IL-13E13K mutation, and certain other mutations which increase affinity for the IL-13R, are also discussed in International Application Publication No. WO 99/51643.

SUMMARY OF THE INVENTION

The invention relates to the uses of mutated forms of IL-13 with higher affinity than that of native IL-13 for the IL-13 receptor. The invention relates to the

discovery that some of the mutants are antagonists of IL-13 mediated activity, and that some are agonists of such activity. These discoveries permit the activity of IL-13 to be upregulated or downregulated according to the particular use intended. Further, the invention relates to the use of certain of the mutants as targeting moieties for immunoconjugates, including immunotoxins directed at cells of cancers which overexpress the IL-13 receptor.

More specifically, the invention provides a method for preventing, or for reducing the severity of, a disease which is mediated by the activity of IL-13, said method comprising administering a mutated IL-13 which is an antagonist of IL-13 activity in an amount effective to prevent, or to reduce the severity of, said disease. In general, the antagonist is a mutated IL-13 in which a glutamic acid residue at position 13 of the IL-13 amino acid sequence is replaced by a neutrally charged or a positively charged amino acid residue. In preferred embodiments, the amino acid substituted for the glutamic acid residue is positively charged. The positively charged residue may be a lysine, an arginine, or a histidine, although arginine is less preferred. Mimetics of the natural amino acids may also be used so long as the mutated IL-13 continues to function as an antagonist of IL-13 activity by, for example, the assays set forth herein. Diseases and conditions that can be prevented or ameliorated by the use of the antagonists include any condition in which IL-13 causes, enhances, mediates, or prolongs the condition. In particular, the disease condition can be bronchial asthma, atopic dermatitis, allergic rhinitis, schistosomiasis, Leishmania, Hodgkin's disease, renal cell carcinoma, Kaposi's sarcoma, or another cancer in which IL-13 serves as a growth factor.

The invention further relates to the use of the antagonists of the invention for the manufacture of a medicament for the prevention or treatment of a disease which is mediated by the presence of IL-13. In a preferred form, the antagonist is a mutated IL-13 in which a glutamic acid residue at position 13 of IL-13 is replaced by a neutrally charged or a positively charged amino acid residue. The medicament can be used for diseases such as bronchial asthma, atopic dermatitis, allergic rhinitis, schistosomiasis, Leishmania, Hodgkin's disease, renal cell carcinoma, Kaposi's sarcoma and any other cancer in which IL-13 functions as a growth factor.

The invention further relates to the discovery that some mutants of IL-13 are agonists of IL-13 activity. The invention therefore provides a method for augmenting

an IL-13-mediated response in a cell, said method comprising contacting said cell with a mutated IL-13, which mutated IL-13 has one or more mutations selected from the group consisting of replacing an arginine residue at position 112 of IL-13 by a neutrally charged or a negatively charged amino acid residue, replacing a glutamic acid residue at position 110 of IL-13 with a neutrally charged or a positively charged residue, replacing an arginine at position 109 of IL-13 with a neutrally charged or a negatively charged residue, replacing a glutamic acid residue at position 92 of IL-13 with a neutrally charged or a positively charged residue, replacing a positively charged residue at position 69 with a neutrally charged or a negatively charged residue, and replacing positively charged residue at position 66 with a neutrally charged or a negatively charged residue, with the proviso that a position of said mutated IL-13 corresponding to position 13 of wild-type IL-13 is not occupied by a lysine residue. In preferred forms, the method includes use of an IL-13 mutated in one or more of the following ways: the arginine at position 112 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid, the glutamic acid at position 110 of IL-13 is replaced by a residue selected from the group consisting of lysine and arginine, the arginine at position 109 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid, the glutamic acid position 92 of IL-13 is replaced by a residue selected from the group consisting of lysine and arginine, a positively charged residue at position 69 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid, or a positively charged residue at position 66 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid. The contacting of the cell occurs *in vitro* or *in vivo*.

The invention further provides for the use of agonists of IL-13 in the manufacture of a medicament for pretreating bone marrow stem cell donors. The mutated IL-13 may have one or more mutations selected from the group consisting of replacing an arginine residue at position 112 of IL-13 by a neutrally charged or a negatively charged amino acid residue, replacing a glutamic acid residue at position 110 of IL-13 with a neutrally charged or a positively charged residue, replacing an arginine at position 109 of IL-13 with a neutrally charged or a negatively charged residue, replacing a glutamic acid residue at position 92 of IL-13 with a neutrally charged or a positively charged residue, replacing a positively charged residue at position 69 with a neutrally charged or a negatively charged residue, and replacing positively charged residue at position 66 with a

neutrally charged or a negatively charged residue; with the proviso that a position of said mutated IL-13 corresponding to position 13 of wild-type IL-13 is not occupied by a lysine residue. In preferred forms, the mutated IL-13 has one or more of the following mutations: the arginine at position 112 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid, the glutamic acid at position 110 of IL-13 is replaced by a residue selected from the group consisting of lysine and arginine, the arginine at position 109 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid, the glutamic acid position 92 of IL-13 is replaced by a residue selected from the group consisting of lysine and arginine, a positively charged residue at position 69 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid, and the arginine at position 66 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid, provided that the glutamic acid residue at position 13 is not replaced by a positively charged residue. In less preferred forms, the glutamic acid may be replaced by a neutrally charged residue.

In another group of embodiments the invention provides an IL-13-receptor binding molecule selected from an IL-13, a circularly permuted IL-13, and a molecule with at least about 85% identity to IL-13, which IL-13-receptor binding molecule has a binding affinity for an IL-13 receptor at least about three times higher than that of wild-type IL-13, and which comprises one or more mutations selected from the group consisting of changing an amino acid corresponding to a glutamic acid at position 110 of IL-13 to a neutrally charged or to a positively charged amino acid, and changing an amino acid corresponding to a glutamic acid at position 92 of IL-13 to a neutrally charged or to a positively charged amino acid. The present invention also provides molecules with increased binding affinity to the IL-13R compared to wild type IL-13. The molecules have at least about 85% identity to IL-13, preferably 90% identity, and more preferably 95% identity. The molecules further bind to the IL-13R with at least about three times the affinity with which wild type IL-13 binds to the receptor and in preferred forms bind with 5 times the affinity. In the most preferred forms, the molecules bind with about 10 times the affinity of wtIL-13. The molecules further comprise one or both mutations selected from the group consisting of changing an amino acid corresponding to a glutamic acid at position 110 of IL-13 to a positively charged amino acid and changing an amino acid corresponding to a glutamic acid at position 92 of IL-13 to a positively charged

carcinoma cell, a glioma cell, a medulloblastoma cell, a head and neck cancer cell, a pancreatic cancer cell, and a Kaposi's sarcoma cell. The method involves contacting the tumor cell with a chimeric molecule comprising an effector molecule selected from the group consisting of a cytotoxin, a radionuclide, a ligand and an antibody. The effector molecule is attached or linked to an IL-13 receptor binding molecule of the invention, as a targeting molecule, to form the chimeric molecule. Preferred cytotoxic effector molecules include *Pseudomonas* exotoxin, *Diphtheria* toxin, ricin and abrin. *Pseudomonas* exotoxins, such as PE38, PE38QQR, PE38KDEL, and PE4E, are particularly preferred. The targeting molecule may be conjugated or fused to the effector molecule with attachment by fusion preferred for cytotoxic effector molecules. The tumor growth that is impaired may be tumor growth in a human. Thus the method may further comprise administering the chimeric molecule to a human by any of various parenteral means, such as administration into a vein, into a body cavity, or into a lumen or an organ. The method may further comprise direct injection or administration into the central nervous system, such as the brain or the spinal fluid.

In yet another embodiment, this invention provides for a method of detecting the presence or absence of a tumor. The method involves contacting the tumor with a chimeric molecule comprising a detectable label attached to an IL-13R binding molecule and detecting the presence or absence of the label. In a preferred embodiment, the label is selected from the group consisting of a radioactive label, an enzymatic label, an electron dense label, and a fluorescent label.

This invention also provides for vectors comprising a nucleic acid sequence encoding a chimeric polypeptide fusion protein comprising an IL-13R binding molecule of the invention, attached to a second polypeptide. The chimeric polypeptide fusion protein specifically binds to a tumor cell bearing an IL-13 receptor. A preferred vector encodes an IL-13R binding molecule-PE or cpIL-13R binding molecule-PE fusion protein. In more preferred forms, the PE moiety is PE38QQR, PE4E, PE38KDEL, or PE4E.

This invention also provides for host cells comprising a nucleic acid sequence encoding a chimeric polypeptide fusion protein comprising an IL-13R binding molecule attached to a second polypeptide. A preferred host cell comprises a nucleic acid encoding an IL-13R binding molecule attached to a PE, such as PE38, PE38QQR, PE38KDEL, or PE4E, and even more preferably encodes them as a fusion protein. The

encoded fusion protein specifically binds to a tumor cell bearing an IL-13 receptor. Particularly preferred host cells are bacterial host cells, especially *E. coli* cells.

In still yet another embodiment, this invention provides chimeric molecules that specifically bind a tumor cell bearing an IL-13 receptor. In one preferred embodiment, the chimeric molecule comprises a cytotoxic molecule attached to an IL-13 receptor binding molecule of the invention. The IL-13 receptor binding molecule may be conjugated or fused to the cytotoxic molecule. In a preferred embodiment, the IL-13 receptor binding molecule is fused to the cytotoxin, thereby forming a single-chain fusion protein. Preferred cytotoxic molecules include *Pseudomonas* exotoxin, *Diphtheria* toxin, ricin, and abrin, with *Pseudomonas* exotoxins (especially PE38, PE38KDEL, PE38QQR, or PE4E) being most preferred.

The invention additionally provides for pharmacological compositions comprising a pharmaceutically acceptable carrier and a chimeric molecule where the chimeric molecule comprises and effector molecule attached to an IL-13 receptor binding molecule. The IL-13 receptor binding molecule and effector molecules may be conjugated or fused to each other. Particularly preferred effector molecules include cytotoxins, labels, radionuclides, drugs, liposomes, ligands, and antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Homology of IL-13 among species.

Homology of mature IL-13 among species calculated using the "PILEUP" function of the of GCG program (*see* Example 2, *infra*). The numbering shown here may differ from that usually used for one or more species due to inserted gaps during the homology pileup. Tyrosine is generally classified as an aromatic acid rather than as a charged one. However, since tyrosine as well as glutamic acid or aspartic acid can make hydrogen ion bonds in acidic conditions, it was considered here as a negatively charged group. Four cysteine residues are completely conserved between the four species. Many charged groups are also conserved.

Figure 2. Effect of wt IL-13 and IL-13R112D on the proliferation of hematopoietic cells. TF-1 [Fig. 2A] and B9 [Fig. 2B] cells were incubated at 37°C with various concentrations of wt IL-13 or IL-13R112D as described in the Examples. Data

are represented as mean cpm of quadruplicate determinations ± SD. The experiment was repeated 9 times.

Figure 3. Effect of wt IL-13 and IL-13R112D on CD14 expression on primary monocytes. Elutriated monocytes were cultured in medium containing 0-50 ng/ml wt IL-13 or IL-13R112D for 48 hr. Cells were then stained with anti CD14-FTC conjugated antibody or isotype control and analyzed for CD14 expression by FACScan equipment.

Fig. 3A. Fluorescence intensity is shown on the x axis as mean channel number on a log scale.

Fig. 3B. Mean fluorescence intensities of each concentration of ligand were shown as a table.

Fig. 3C. Mean fluorescence intensities of each ligand were shown as a graph. The suppressive effect of CD14 with 1 ng/ml of IL-13R112D is seen to be comparable to that of 10 ng/ml of wt IL-13.

Figure 4. Inhibition of binding of [^{125}I]IL-13 and [^{125}I]IL-4 on PM-RCC cells.

Cells (1 x 10^6) were incubated with 500 pM [^{125}I]IL-13 [Fig. 4A] or 500 pM [125^{125}I]IL-4 [Fig. 4B] with or without increasing concentrations (up to 10 nM) of wt IL-13 or IL-13R112D. Bound radioactivity was determined as described in the Examples. Data are presented as the mean percentage of maximal specific binding without unlabeled ILs. Total ^{125}I-IL-13 [Fig. 4A] and ^{125}I-IL-4 [Fig. 4B] bound to PM-RCC cells was 2962 ± 123 and 1706 ± 141, cpm ± SD, respectively. Data are represented as mean ± SD of duplicate determination. The SD are shown when they are larger than the symbols.

Figure 5. Cytotoxicity of IL-13PE38 on PM-RCC cells.

Figure 5A. Cells were cultured with various concentrations of IL-13PE38 (0, 0.1 -1000 ng/ml) with or without 2000 ng/ml of wt IL-13 or IL-13R112D.

Figure 5B. Cells were cultured with various concentrations of wt IL-13 or IL-13R112D (0, 0.2-2000 ng/ml) with IL-13PE38 as described in the Examples. The data were obtained from the mean of quadruplicate determinations, and the assay was repeated

several times. The concentration of IL-13-PE38 at which 50 % inhibition of protein synthesis occurred (IC50) was calculated. Data are means; bars represent the SD.

Figure 6. SDS-PAGE analysis of purified human IL-13 and its mutants.

Approximately five hundred nanograms of each purified cytokine was loaded per sample. Proteins were detected using a Coomassie Blue stain. M; MultiMark™ Multi-Colored Standard (Novex, San Diego, CA).

Figure 7. Competition for the binding of ^{125}I-IL-13 by wtIL-13 and double mutein IL-13E13KR112D.

$2x10^5$ U251 cells were incubated with 500 pM ^{125}I-IL-13 with various concentration of unlabeled wtIL-13 or IL-13E13KR112D. The cell-bound radioactivity was determined with a gamma counter. The error bars represent the standard deviation of duplicate determinations.

Figure 8. Proliferation or TF-1 cells induced with human IL-13 or its mutants.

Panel A. Ten to twenty thousand TF-1 cells per well were cultured in the presence or absence of various concentration of wtIL-13 or its mutants for 52 - 54 h.

Panel B. The cells were cultured with or without 3 ng/ml wtIL-13 and presence or absence of 300 ng/ml or 1μg/ml E13KR112D. The cell proliferation was determined by the uptake of 3H-thymidine in the dividing cells. The reported data are the average of triplicate or quadruplicate samples with error bars representing the standard deviation within a data set. Experiments were repeated several times. DM: double mutein, IL-13E13KR112D. * represent statistically significant at $p < 0.01$ level from no IL-13 control; ** $p < 0.01$ vs. wtIL-13; *** not significant vs. no IL-13 control and $p < 0.01$ vs wtIL-13.

Figure 9. Down modulation of CD14 expression on monocytes

Primary elutriated monocytes ($1x10^7$ /tube) were cultured with or without 1 ng/ml wtIL-13 in presence or absence of 1μg/ml E13KR112D for 48 hr. Then cells were washed and stained as described in Example 16. Gated mean fluorescence intensity (MFI) number are indicated in each panel.

Figure 10. Activation of STAT6 by IL-13 or its mutants in various cell lines.

Panel A. Photos of gels showing results of EBV-B, THP-1 or KSY-1 cells incubated with 0, 1, 10 or 50 ng/ml wtIL-13 or its mutants for 15 minutes.

Panel B. THP-1 cells were incubated with 0 or 10 ng/ml wtIL-13 or with 10 ng/ml wtIL-13 and 100 or 500 ng/ml IL-13E13KR112D for 15 minutes.

WtIL-13 and IL-13E13KR112D were added simultaneously. Position of STAT6 on each gel is shown by a line (panel A) or by an arrowhead (panel B). Cells were processed and electrophoretic mobility-shift assay (EMSA) were performed as described in Examples 16 and 17.

Figure 11. IL-13 and double mutein block the cytotoxic activity of IL-13PE38QQR on U251 cells and PM-RCC cells. A thousand cells per well were cultured in leucine-free media. All cultures were incubated over night prior to the addition of 1 μCi tritiated leucine. Cells were then incubated for 4 h, harvested and radioactivity counted with beta counter. The data are the average of quadruplicate determinations with the error bars representing the standard deviation within a data set. Experiments were repeated twice.

Panel A. PM-RCC cells in various concentration of an IL-13-immunotoxin (IL-13PE38QQR) and 1 μg/ml wtIL-13 or its muteins. The legend in panel A refers to the wells in which a competitor was present (the three data lines at the top of the panel). A fourth line, with open triangles denoting the data points, shows the results for cells exposed to the IL-13 immunotoxin in the absence of wtIl-13 or its muteins, as denoted by the legend "No competitors." Y-axis is ^{3}H-Leucine incorporation in counts per minute (CPM).

Panel B. Graphs of U251 cells (top graph of panel) and PM-RCC cells (bottom graph of panel) in the presence of 1 ng/ml IL-13PE38QQR with or without various concentrations of wtIL-13 or of its mutants.

Figure 12. SDS-PAGE analysis of purified IL-13 and its mutants.

Approximately five hundred nanograms of each purified cytokine was loaded per sample. Proteins were detected using Coomassie Blue stain. M; MultiMark™ Multi-Colored Standard (Novex, San Diego, CA). Legend: wt IL-13: wild-type IL-13. E13K: IL-13E13K. R112D: IL-13R112D.

Figure 13. Competition for the binding of ^{125}I-IL-13 by wtIL-13 and IL-13E13K

$5x10^5$ PM-RCC cells (shown in panel A) or U251 cells (shown in panel B) per tube were incubated with 200 pM or 500 pM ^{125}I-IL-13, respectively, and with various concentrations of unlabeled wtIL-13 or IL-13E13K. Data presented are total cell bound ^{125}I-IL-13 with error bars representing the standard deviation of duplicate determinations.

Figure 14. Proliferation of TF-1 cells induced by IL-13 or IL-13E13K.

Panel A. Ten thousand TF-1 cells per well were cultured in the presence or absence of various concentration of wtIL-13 or IL-13E13K for 52 - 54 h.

Panel B. The cells were cultured with or without 1 μg/ml IL-13E13K and various concentrations of wtIL-13 for 52 h.

Panel C. The cells were cultured with or without 3 ng/ml wtIL-13 and in the presence or absence of 300 ng/ml or 1μg/ml IL-13E13K.

For all panels, cell proliferation was determined by the uptake of ^{3}H-thymidine in the dividing cells. Data presented are percentage of count without cytokine stimulation and the average of triplicate or quadruplicate samples with error bars representing the standard deviation within a data set. Experiments were repeated several times. E13K: IL-13E13K.

Figure 15. Effect of IL-13E13K on IL-13 induced down modulation of CD14 expression on monocytes

Primary elutriated monocytes ($1x10^7$ /tube) were cultured with or without 1 ng/ml wtIL-13 in the presence or absence of 1μg/ml IL-13E13K (shown as "E13K") for 48 hr. Cells were then washed and stained as described in material and methods. Gated mean fluorescence intensity (MFI) number is indicated in each panel.

Panel A: Cells cultured without cytokine stimulation.

Panel B: Cells cultured in presence of wtIL-13, but absence of IL-13E13K.

Panel C: Cells cultured in presence of wtIL-13 and in presence of IL-13E13K.

Figure 16. Activation of STAT6 by IL-13 or *IL-13E13K* in various cell lines.

Panel A: EBV-B, THP-1 or KSY-1 cells were incubated with 0, 1, 10 or 50 ng/ml wtIL-13 or IL-13E13K for 15 minutes.

Panel B: THP-1 cells were incubated with 0 or 10 ng/ml wtIL-13 or with 10 ng/ml wtIL-13 plus 100 or 500 ng/ml IL-13E13K for 15 minutes.

For both panels, wtIL-13 and IL-13E13K were added simultaneously. Cells were processed and EMSA was performed as described in Example 18. Location of STAT6 on each gel is noted.

Figure 17. Predicted model for IL-13 interaction with its receptors. Homology model of the CRH domain of IL-13Rα', IL-4Rα chains and IL-13. This model is designed to show the type II IL-13R complex. The 3-dimensional model is shown as a ribbon diagram. A and D indicate α-helix A and D of the IL-13 molecule, respectively. The figure was prepared using the InsightII program.

Figure 18. Suppression of HD cell proliferation by an exemplary IL-13 antagonist.

Panel A. L1236 cells ($2X10^4$/well) were cultured in RPMI with or without 2 μg/ml IL-13E13K (Antagonist) in a CO_2 incubator for 48 hr or 72 hr. Viable cells were counted after trypan blue exclusive staining (Life Technologies, Inc., Grand Island, NY). Data is shown as mean ± standard deviation of cell number per well. * $p < 0.01$

Panel B. $1x10^5$/ml L1236 cells (left two bars) or L428 cells (right two bars) were cultured with or without 7.5 μg/ml IL-13 antagonist for 48 hr in 37°C humidified 5% CO_2 incubator. After 1μCi ^{3}H-thymidine pulse per well, cells were incubated for additional 9 hr and then harvested. Data is shown as mean ± standard deviation of CPM. Symbol: **; $p = 0.03$, N.S.; not significant.

Panel C. $1x10^5$/ml L1236 or L428 cells were incubated in RPMI containing 0.1 ng/ml wild-type IL-13 with or without various concentration of IL-13 antagonist for 48 hrs in a 37°C, humidified, 5% CO_2 incubator. After 1μCi ^{3}H-thymidine pulse per well, cells were incubated for additional 9 hrs and then harvested and counted. Data is shown as mean ± standard deviation of percent CPM.

Panel D. $1x10^5$/ml L1236 or L428 cells were incubated in RPMI containing 0.1 ng/ml wild-type IL-13 with or without various concentration of IL-13-BP. Remainder of protocol was as in Panel C.

Figure 19. Expression of IL-13 and functional receptor complex in Hodglin's disease cell lines.

Panel A. Rt-PCR assay was performed as described in Example 20. The rt-PCR reaction was performed as follows: initial reverse transcription step at 48°C for 45 min followed by inactivation of reverse transcriptase and denaturation step at 94°C for 2 min; 40 cycles at 94°C 30 sec; 54°C 60 sec, 68°C 120 sec, respectively. The products were electrophoresed in 1% agarose gel and stained by ethidium bromide.

Panel B. Dot blot analysis for secretion of IL-13 protein by HD cell lines.

Panel C. Growth of L1236, L591, and L428 HD cell lines in absence or in presence of IL-13.

Panel D. Growth of L1236 or L428 HD cells in absence or in presence of immunotoxin IL-13-PE38QQR.

DETAILED DESCRIPTION

I. Introduction

The invention provides methods of modulating the effect of IL-13 on cells and on illnesses. First, the invention concerns the fact that certain mutants of IL-13 which have higher binding affinity for the IL-13 receptor ("IL-13R") than does IL-13 function as antagonists of IL-13 activity, while other mutants act as strong agonists of IL-13. Use of the agonists permits the activity of IL-13 to be upregulated when that is desirable, such as when activating dendritic cells *in vitro*, while use of the antagonists permits the activity of IL-13 to be downregulated when that effect is desirable, such as decreasing the effect of IL-13 in inflammatory diseases such as asthma. Additionally, the invention provides new mutants with a high affinity for the IL-13R. These mutants can be fused to an effector molecule to form a chimeric molecule known as an immunoconjugate. Where the fused effector molecule is a toxin, the construct is known as an immunotoxin; immunotoxins targeted by the high affinity IL-13 mutants of the invention can be used to specifically kill cells overexpressing the IL-13R. Since the cells of many cancers, such as gliomas, overexpress the IL-13R, the new mutants offer new

immunotoxins with which to attack these cells. The mutants can be used, for example, to purge cell cultures of IL-13-overexpressing cancer cells.

The discussion below first discusses IL-13 mutants which have higher binding affinity for the IL-13R than that of IL-13. The mutants comprise changes of the glutamic acid ("E", in single letter code) at position 110 of IL-13, or the glutamic acid at position 92 of IL-13, or both, to an amino acid which is positively charged at physiological pH, such as lysine or arginine.

The discussion then discusses the biological activity of mutants of IL-13. In particular, mutants of IL-13 in which the arginine at position 112 is altered to a neutral amino acid or, more preferably, to an acidic amino acid, such as glutamic acid or aspartic acid, are strong agonists of IL-13 activity. That is, such mutants activate IL-13 mediated activity more strongly than does native (also called "wild-type" or "wt") IL-13. Mutating the arginine at position 112 to aspartic acid has previously been suggested to create a mutant which binds more specifically to the IL-13R, rendering the mutant useful as the targeting portion of an immunotoxin directed to IL-13-expressing cells. The increased biological activity of the mutant and its utility by itself as an agent, independent of the activity of an effector molecule fused to it, have not previously been identified.

The discussion then turns to the discovery that mutating the glutamic acid ("E") at position 13 to a neutral amino acid, or more preferably an amino acid which carries a positive charge at physiological pH, results in a mutant that is an antagonist of IL-13. That is, in the presence of such a mutant, the activity of endogenous IL-13 is reduced or wholly blocked. This permits the alleviation of conditions in which IL-13 is implicated as a causative or enhancing agent. Such conditions include, for example, asthma, allergic rhinitis, certain cancers, such as Hodgkin's Disease, Kaposi's sarcoma , and renal cell carcinoma, and susceptibility to Leishmaniasis. Remarkably, the presence of a mutation to a neutral amino acid or, more preferably, to a basic acidic acid at position 13 of IL-13 causes the mutant to be an antagonist of IL-13 activity even if the molecule contains other mutations, such as changing the arginine at position 112 to aspartic acid, which would otherwise cause the mutant to be a strong agonist of IL-13 activity. For example, the double mutant IL-13E13KR112D is an antagonist of IL-13 activity even though the mutant IL-13R112D is a strong agonist of IL-13-mediated activity.

A. Molecules with Increased Affinity for the IL-13 Receptor

As noted in the Background, the crystal structure of IL-13 is not known. IL-13 does, however, have some homology to IL-4 and binds to one or more chains of the IL-4 receptor ("IL-4R"). In the absence of direct information, inferences about the structure and binding positions of IL-13 are made by analogy to the structure of IL-4. Previous work has shown that mutating a residue of glutamic acid in human IL-4 (hIL-4) at position 9 to lysine severely impaired the binding of hIL-4 to the IL-4α chain. Kruse *et al.*, *EMBO J* 12:5121-5129 (1993). This residue is conserved between IL-4 and IL-13 (in which the residue is predicted to fall at position 13), and it was thought the residue might be involved in binding of hIL-13 to chains common to both the IL-13R and the hIL-4R. As reported in Debinski *et al.*, 1998, mutating this residue to lysine (that is, changing a negatively charged residue to a positively charged residue), increased its binding affinity of the mutated IL-13 to hIL-13R, but decreased the ability of the interleukin to stimulate the growth of cells exposed to it.

Based on the crystallographic structure of IL-4 and the homology of the amino acid sequences, it has been predicted that IL-13 has four alpha helices (styled alpha helices A, B, C, and D) and two beta sheets. *See*, Bamborough, P. *et al.*, *Protein Engineering* 7:1077-1082 (1994). The residue mutated in the work reported in Debinski *et al.*, 1998, was in the putative A alpha helix of the molecule. Debinski did not report examination as to the effect of mutating residues in other helices of IL-13.

Mutating the charge of a different residue in a different putative helix of IL-13, the arginine at position 112 in helix D, results in a molecule with a sharply increased affinity for the IL-13R compared to wtIL-13. In competitive binding assays, the mutated IL-13 (IL-13R112D) showed an affinity for the IL-13R some 5 to 10 times greater than that of wild type IL-13 (wtIL-13). *See, e.g.*, Figure 4 and Examples 7 and 13. In preferred embodiments, the arginine at position 112 can be mutated to a neutral amino acid. In more preferred embodiments, it can be mutated to an aspartic acid or a glutamic acid, with aspartic acid being the most preferred. Other negatively charged amino acids or mimetics can, however, be used. Any construct or derivative which does not bind to the IL-13 receptor with at least about 3 times the affinity of wtIL-13 is not within the scope of the claimed invention.

Because the results noted above demonstrate that amino acid residues in the putative D helix of IL-13 are involved in interactions with the IL-13R, changing the

charge of two other, nearby charged residues predicted to be in helix D, arginine at position 109 and the glutamic acid at position 110, will likewise result in increased affinity for the IL-13R. The preferred mutations for the arginine at position 109 are the same as those discussed above with regard to the arginine at position 112. With respect to the glutamic acid at position 110, since glutamic acid is negatively charged at physiological pH, the preferred mutations are those which replace the glutamic acid residue with a neutral or with a positively charged amino acid. In preferred embodiments, the glutamic acid is mutated to a positively charged residue such as a lysine or an arginine, although other positively charged natural amino acids, such as tyrosine, synthetic amino acids, or mimetics can be used, so long as the resulting molecule retains at least about 3 fold higher binding affinity for the IL-13 receptor compared to wild type IL-13.

Based on studies of residues conserved among species, the consideration that charged residues are more likely to be exposed on the surface of the protein, and a review of the chains which are shared between the IL-4 and the IL-13 receptors and those which are not, changing the negatively charged glutamic acid at position 92 of IL-13 to a neutral residue or to a positively charged residue will also increase its binding to the IL-13R. In preferred embodiments, the amino acid substituted for the glutamic acid at position 92 is a lysine or an arginine, although other positively charged natural amino acids, such as tyrosine, synthetic amino acids, or mimetics can be used, so long as the resulting molecule retains at least about 3 fold higher binding affinity for the IL-13 receptor compared to wild type IL-13.

It is understood that variations of IL-13 can be made which retain the capacity to bind to the IL-13 receptor but which do not follow the amino acid sequence of wild type IL-13. In the simplest form, for example, a molecule might be made which contains one, two, or more conservative substitutions, particularly at positions not expected to be implicated in binding to the IL-13 receptor. Such variations are intended to be encompassed within the invention. Specifically, the invention encompasses IL-13 receptor binding molecules with at least about 85% identity to IL-13 and which have about 3 times the binding affinity for the IL-13 receptor of wild type IL-13 or more and which have a charge-changing mutation at one, two, three, or all four of the positions specified above. More preferably, the IL-13 receptor binding molecules have at least about 90 % identity to IL-13 and, even more preferably, have about 95% identity to the wild type sequence. In the most preferred embodiments, the IL-13 receptor binding

molecules are wild type IL-13 with a charge-changing mutation at residue 112, residue 110, residue 109, residue 92, or any two of these positions, or at three, or at all four.

The numbers of the residues set forth above are to the accepted sequence for mature human IL-13 as shown in Figure 1. As discussed in more detail below, however, IL-13 can be permuted to create so-called circularly permuted IL-13, or "cpIL-13." Further, the sequence of native IL-13 can be altered by, for example, removing terminal residues, conjugation, or the like. Each of these variations can change the numbering of the residues. It is therefore understood that reference herein to residues "112," "110," "109" and "92," refers in its first sense to the amino acid residues in those position of the residues in mature wild-type human IL-13, as set forth, for example, in Figure 1, but also refers, as appropriate in context, to residues in variations of IL-13 or cpIL-13 which correspond to residues 112, 110, 109, and 92 of the mature, wild-type IL-13 sequence, so long as the mutation of the corresponding residues results in at least about a 3 fold increase in binding affinity of the molecule to the IL-13 receptor compared to the binding affinity of IL-13. To clarify that these higher binding affinity molecules are not necessarily merely wild type IL-13 with mutations in these particular residues, the molecules provided by the invention have been termed "IL-13 receptor binding molecules."

B. Higher Affinity IL-13R Binding Molecules Can Also Have Increased Biological Activity

In marked contrast to the results reported in Debinski *et al.*, 1998, in which the mutated IL-13 lost virtually all biological activity, IL-13R112D showed increased, not decreased, biological activity. As discussed in Examples 10, 11, and 12, below, the mutated IL-13 showed approximately 10-fold higher activity than did wtIL-13 in standard assays for the activity of this interleukin. In this regard, IL-13R112D showed 10-fold higher activity in the inhibition of CD14 expression in primary monocytes, and 10-fold higher activation of STAT6 on EBV-immortalized B cells and the THP-1 monocytic cell line. *See*, Figure 3 and Example 12, *infra*. STAT6 is a signal transduction molecule and activator of transcription which is known to be activated by IL-13. Whereas the mutation reported by Debinski *et al.*, 1998 showed a marked *decrease* in the ability of the interleukin to induce proliferative activity, IL-13R112D induced a 10-fold *increase* in proliferation of TF-1 and B9 cell lines. *See*, Figure 2A and B. And, IL-13R112D

interacted with the IL-13 receptor with greater activity than wtIL-13 in all cell types tested.

Given these results with the mutation of arginine to the negatively charged aspartic acid residue, it is expected that mutating the arginine to a residue with a neutral charge or to another residue with a negative charge at physiological pH, such as glutamic acid, will likewise result in an IL-13 mutant with an activity as an agonist stronger than that of native IL-13. In more preferred embodiments, the residue substituted for the arginine at position 112 is negatively charged. Any particular residue or mimetic can be tested for its effect as an agonist by the assays set forth in the Examples below. Aspartic acid is the most preferred residue to substitute for the arginine at position 112.

As noted in the Background section, IL-13 is a potent activator of the immune system. In particular, IL-13 is an activator of dendritic cells, which are professional antigen-presenting cells ("APCs"), and can be used, in conjunction with GM-CSF, to cause differentiation of monocytes into dendritic cells. Due to the increased biological activity of the IL-13 receptor binding molecules of the invention, they can be substituted for IL-13 or IL-4 in *in vitro* protocols to activate dendritic cells or other APCs. While APCs activated by these protocols have several uses, in one important embodiment, they are often used in procedures to "pulse" or to "load" dendritic cells with antigen prior to reinfusing the APCs into a patient to augment or to induce an immune response against that antigen.

The agonists of the invention can also be used in vitro to reduce the occurrence of Graft Versus Host Disease ("GVHD"). GVHD is a significant concern after allogeneic stem cell transplantation. Various studies have demonstrated that type-1 T lymphocytes (secreting interleukin("IL")-2 and interferon-gamma) in harvested donor cells mediate acute GVHD, whereas type-2 T lymphocytes (secreting IL-4 and IL-10) can prevent acute GVHD. Type-2 T cells also produce IL-13. It is clinically well known that pretreatment of donors with granulocyte colony-stimulating factor (G-CSF) suppresses the severity of GVHD even though there are many lymphocytes contaminants in the harvested stem cells of the G-CSF-treated donor. This is because pretreatment of donors with G-CSF polarizes the donor's T lymphocytes toward type-2 cytokine production. In murine models, this has been shown to reduce severity of experimental GVHD (see, e.g., Blood, 86(12):4422-4429 (1995)). As wild type IL-13 is shown to polarize type-2 T cells, it is predicted that IL-13R112D may more powerfully polarize type-2 T cells of donors resulting in prevention of acute GVHD.

C. IL-13E13KR112D is a powerful IL-13 antagonist

The double mutant IL-13E13KR112D produced by site-directed mutagenesis of two amino acids in the predicted α helix A and D of the IL-13 molecule, is an antagonist of IL-13 activity. The antagonistic activity of IL-13 mutein IL-13E13KR112D was determined based on the inhibition of wtIL-13 induced proliferation of TF-1 cells, wtIL-13 induced down modulation of CD14 in primary monocytes, inhibition of wtIL-13 induced activation of STAT6 in B cells, monocytes and cancer cells, displacement of ^{125}I-IL-13 binding on cancer cells and neutralization of cytotoxicity mediated by IL13-PE38QQR in cancer cells. Thus, the antagonistic activities of IL-13E13KR112D were evident in cells that expressed Type I and type II/III IL-13 receptors.

In an initial study, reported in Section I B, above, a single IL-13 mutein, IL-13R112D, was created by substituting aspartic acid (D) for arginine (R) at position 112. Because the position of amino acid 112 in IL-13 was predicted to correspond to position 124 in the IL-4 molecule, and since mutation of position 124 of IL-4 resulted in an IL-4 antagonist (IL-4Y124D) (Kruse, N. et al., Embo J. **11**:3237-3244 (1992)), it was predicted that IL-13R112D would be an IL-13 antagonist. Surprisingly, the opposite effect was observed. IL-13R112D turned out to be a powerful IL-13 agonist with 5-10 fold improved binding affinity to IL-13 receptors, as reported in Section I B.

A mutation at position 13 of IL-13, which is predicted to be located in the α helix A of the IL-13 molecule has also been produced. In this IL-13 mutein, the glutamic acid at position 13 was changed to lysine (Debinski, W. et al., *Nat. Biotechnol.* **16**:449-453 (1998)). This mutant protein, IL-13E13K, reported 50-fold higher binding affinity to IL-13 receptors compared to wtIL-13 on U251 cell lines. *Id.*. To further improve the binding affinity of IL-13, a double mutant, IL-13E13KR11D, was produced. It was predicted that this molecule would have a much higher binding affinity to IL-13 receptor compared to wtIL-13, IL-13R112D and IL-13E13K. This molecule was expressed in *Escherichia coli* and homogeneously purified material was found to bind IL-13 receptors. Surprisingly, this double mutein IL-13 when tested in various biological assays turned out to be an antagonist of IL-13, rather than an agonist. Based on this result, an IL-13 mutated to a neutral or to a negatively charged residue at position 112 are agonists unless the glutamic acid residue at position 13 has been mutated to a positively charged residue.

D. IL-13E13K is also a powerful antagonist of IL-13 activity

IL-13E13K, a powerful antagonist of IL-13, which bound well to the IL-13R, was produced by site-directed mutagenesis of an amino acid in the predicted α-helix A of the IL-13 molecule. Without wishing to be bound by theory, the results demonstrate that Glu13 in IL-13 is of crucial importance for the potency of the cytokine for signal generation, but not for the binding of IL-13 to its receptors. The antagonistic activity of IL-13 mutein *IL-13E13K* was determined based on: a) 4 to 8-fold better displacement of ^{125}I-IL-13 binding on cancer cells; b) the inhibition of wtIL-13 induced proliferation of TF-1 cells; c) neutralization of wtIL-13 induced down modulation of CD14 expression in primary monocytes; and d) inhibition of wtIL-13 induced activation of STAT6 in B cells, monocytes and cancer cells. Thus, the antagonistic activities of *IL-13E13K* were evident in cells that expressed Type I, Type II and Type III IL-13 receptors.

In view of this result, and those reported above for the double mutant IL-13E13KR112D, it is evident that mutating the acidic residue at position 13 causes the mutant to be an antagonist of IL-13 activity. Thus, mutants of IL-13 in which the glutamic acid at position 13 is changed to a residue with a neutral charge will also act as antagonists of IL-13 activity. In preferred embodiments, the glutamic acid at position 13 is changed to a residue which is positively charged at physiological pH. For example, the glutamic acid residue at position 13 can be mutated to lysine, arginine or histidine. Arginine is somewhat less preferred as a positively charged residue; in more preferred embodiments, the glutamic acid is mutated to lysine or histidine. A mutation to lysine is the most preferred embodiment.

IL-13 receptors belong to a large family of cytokine receptors, including receptors (or receptor subunits) for interleukins-2, 3, 4, 5, 6, granulocyte macrophage colony-stimulating factor, granulocyte colony-stimulating factor, erythropoietin, growth hormone and prolactin (Bazan, J. F. *Proc Natl Acad Sci, USA* **87**:6934 (1990)). All these proteins show particular homology in the amino acid sequence of their postulated or proven ligand binding CRH domains. These receptors also belong to the immunoglobulin superfamily which consists of many proteins characterized by immunoglobulin-like beta sandwich structure. *Id.* Despite similarities in receptors, their cognate ligands show only limited homology at the amino acid sequence level. However, crystallographic analyses or computer prediction of the topology of ligands indicate that they have similar tertiary

structure in that they are composed of four major α-helixes arranged in up-up & down-down directions. Based on these studies, the critical interface residues for IL-4 were found to be located in α-helix A and D of the ligand. Similarly, the amino acid residue which is critical for receptor binding and signal transduction for murine GM-CSF is reported to be Glu21 which is located in α-helix A (Altmann, S. W. et al., *J Biol Chem* **270**:2233 (1995); Altmann, S. W. et al., *Growth Factors* **12**:251 (1995)). The receptor binding domains of growth hormone and interleukin-6 are reported to be located in α-helix A and C (DeVos, A. M. et al., *Science* **255**:306 (1992); Savino, R. et al., *EMBO Journal* **13**:1357 (1994); Savino, R. et al., *EMBO Journal* 13:5863 (1994)). Without wishing to be bound by theory, our studies suggest that residue Arg112, predicted to be located in the C-terminal end of α-helix D of hIL-13, is one of the critical residues of receptor binding. The results reported here suggest that residue Glu13, predicted to be located in α-helix A, is also critical for receptor interaction. Based on these studies, we predict that the receptor interface of IL-13 may be located in α-helix A and D. These predictions are supported by the proposed model of IL-13 and IL-13R complex. Thus, this model may be an useful tool to investigate receptor binding sites in the IL-13 molecule.

Since IL-13 has been shown to be involved in many inflammatory diseases including bronchial asthma, it is expected that IL-13E13K and IL-13E13KR112D and other antagonists of the invention will be able to neutralize or reduce the effect of IL-13, reducing the severity or occurrence of asthma symptoms. Similarly, since IL-13 is involved in allergic rhinitis and atopic dermatitis, it is expected that IL-13E13K, IL-13E13KR112D and other antagonists will be useful in mitigating these conditions.

E. Antagonists to IL-13 Slow the Growth of Hodgkin's Disease, Renal Cell Carcinoma, and Kaposi's Sarcoma cells

Cytokines may play a critical role in the survival and proliferation of Hodgkin/Reed-Sternberg ("H/RS" or "HD/RS") cells. Histopathological studies have demonstrated that a large number of (reactive) lymphocytes and some eosinophils surround H/RS cells. The symptoms such as fever, night sweat or weight loss seen in Hodgkin's Disease ("HD") patients have been hypothesized to be caused by cytokines produced by H/RS cells themselves or surrounding cells (Drexler, HG, *Leukemia and Lymphoma,* 8:283-313 (1992) ("Drexler 1992"); Drexler, HG, *Leukemia and Lymphoma,*

9: 1-25 (1993) ("Drexler 1993")). While as much as thirteen cytokines were postulated to be autocrine/paracrine growth factors for H/RS cells, it has not been clarified which cytokine(s) is essential for pathogenesis of HD (Drexler, 1992; Drexler 1993).

Recently by using microarray technology it was discovered that IL-13 might be an autocrine growth factor for H/RS cells (Kapp et al., *J Exp Med,* 189:1939-1945 (1999)). In two cell lines tested, one cell line grew in autocrine fashion and neutralizing IL-13 monoclonal antibody blocked the proliferation of these cells. *Id.* The mechanism of selective inhibition of cell growth was not demonstrated.

As reported above, the present invention provides IL-13 mutants with altered bioactivities, such as antagonist or super agonist properties. In particular, a novel IL-13 antagonist, *IL-13E13K*, in which glutamic acid at position 13 was substituted by lysine in the IL-13 molecule, was produced. This mutant binds to IL-13R with up to 8 fold higher affinity than that of wild-type IL-13. Moreover, it antagonizes the proliferative activity of IL-13 on TF-1 erythroleukemia cells, reverses the IL-13 induced down modulation of CD14 expression on monocytes and suppresses the IL-13 induced STAT-6 activation in Ebstein-Barr virus immortalized B-cells, THP-1 monocytic cell line and in KSY-1 Kaposi's sarcoma cells. Because IL-13 has been shown to be autocrine growth factor on H/RS cells, the IL-13 antagonist was examined to determine whether it could inhibit the autocrine/paracrine proliferation of these cells. In addition, the subunit composition of IL-13R was examined to determine why some H/RS are responsive to IL-13 and IL-13 antagonists.

All H/RS cell lines express mRNA for IL-13R components but only one of two-expressed functional IL-13R as determined by IL-13 induced proliferation and internalization. It is of interest to note that in the previous study, suppression of IL-13 effect using antibody was seen in only one of two H/RS cell lines even though both cell lines expressed IL-13 (Kapp et al., 1999, *supra*). Thus, the Kapp 1999 study confirms that some H/RS cells may not express functional IL-13R.

The studies reported in the Examples also indicate that IL-13-PE38QQR can be used for the eradication of H/RS cells *in vitro* and *in vivo*. The concentration of IL-13-PE38QQR that causes maximal inhibition of protein synthesis is clinically achievable. Preclinical studies have suggested that high serum level of IL-13PE38QQR can be safely achieved in monkeys when given intravenously (Cmax; 5027 ± 1583 ng/ml). Thus, sensitivity of H/RS cell may be within a therapeutic range *in vivo*.

The mechanism(s) of different sensitivity of H/RS cells to IL-13 and IL-13 antagonist is still not clear. The lack of response to IL-13 on L428 may be explained by low level expression of IL-13Rα2 chain. IL-13 Ra2 chain has been shown to bind to IL-13 with high affinity but it does not signal by itself. Similarly, cell lines that express IL-13Rα2 chain do not seem to respond to stimulatory growth effect of IL-13. Alternative mechanism(s) may also be operational for example, disbalanced expression of different receptor chains, inappropriate receptor subunits aggregation or mutation of receptor chains.

Taken together, the results show that *IL-13E13K* is a useful agent for the therapy of HD in which H/RS cells express functional IL-13R. The results with IL-13E13KR112D indicate that the presence of the E13K mutation, or the mutation of E13 to a neutral residue or, preferably, to arginine, will likewise result in an IL-13 antagonist. The antagonists of the invention are superior to anti-IL-13 antibody (Kapp et al., 1999, *supra*) because the IC_{50} of antibody is 1000 times higher than that of *IL-13E13K*. The antagonists of the invention are also better than using the α chain of the IL-13R (sometimes called the "IL-13 binding protein," or "IL-13BP", see, Zhang et al., J. Biol. Chem. 272: 9474-9480 (1997)). IL-13BP comprises a soluble extracellular domain of the IL-13 receptor which is normally membrane bound. In order to produce the domain in a form in which it can be secreted by recombinant cells, it is fused to the Fc portion of human immunoglobulin. The resulting IL-13Rα/Fc chimera has been found to block the effect of IL-13 in vitro and to block the effect of IL-13 in vivo in animal models of inflammatory disease. The antagonists of the invention are smaller than IL-13BP fusion constructs, and therefore achieve better biodistribution and better bioavailability. Accordingly, they are better agents for blocking IL-13 effects both *in vitro* and *in vivo*.

In recent studies, we have found that Kaposi's sarcoma cells secrete IL-13. Studies with antibodies to IL-13 showed that blocking IL-13 with antibodies inhibited the growth of Kaposi's sarcoma cells, therefore showing that IL-13 functions as a growth factor for these cells. Since the antagonists of the invention react specifically with the IL-13R and bind with at least an order of magnitude higher affinity than antibodies bind to a typical antigen, the antagonists are expected to strongly inhibit the growth of Kaposi's sarcoma cells. Similarly, renal cell carcinoma cells have been found to secrete IL-13 and can be inhibited in the same fashion. Thus, IL-13E13K, IL-13E13KR112D, and other

antagonists of the invention can be used to slow or stop the growth of Kaposi's sarcoma and renal cell carcinoma cells.

The fact that IL-13 serves as an autocrine factor in three such disparate cancers suggests that IL-13 is an autocrine growth factor for other cancers, and particularly those which overexpress the IL-13 receptor. Based on the results above, we expect that IL-13 antagonists can be used to slow the growth other cancers for which IL-13 serves as an autocrine factor. We have recently discovered that some pancreatic cancers and approximately one-quarter of head and neck cancers overexpress the IL-13 receptor. Growth of these IL-13R-overexpressing cancers can be slowed by contacting them with the antagonists of the invention.

II. Definitions

Units, prefixes, and symbols are denoted in their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation. The headings provided herein are not limitations of the various aspects or embodiments of the invention, which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification in its entirety.

As used herein, "wild type IL-13" and "native IL-13" are synonymous and refer to the mature form of IL-13. The nucleotide and amino acid sequences of IL-13 have been publicly available since at least 1993. *See*, McKenzie, *et al.*, Proc Natl Acad Sci USA 90:3735-3739 (1993). The amino acid and nucleotide sequences are available from GenBank under Accession No. L06801. *See also*, SEQ ID NO:1 and discussion in section VI A, *infra*.

The term, "modulating," when used in the context of the biological activity of a molecule, means to upregulate or to downregulate the biological activity of the molecule as desired to achieve an intended end, such as a therapeutic result. Thus, as used herein, modulating the activity of IL-13 refers to increasing or decreasing the effect normally caused by IL-13 in a particular context by use of an agent which acts as an agonist or an antagonist of IL-13.

As used herein, the term "mediate" in connection with the effect of IL-13 on a condition or disease, means that IL-13 causes, contributes to, aggravates, enhances, or prolongs the existence or severity of the condition or disease.

"Negatively charged," in reference to an amino acid, refers to those amino acids, amino acid derivatives, amino acid mimetics and chemical moieties that are negatively charged at physiological pH. Negatively charged amino acids include, for example aspartic acid and glutamic acid, which are preferred negatively charged amino acids for use in the invention. An "acidic" residue is a residue that is negatively charged at physiological pH.

"Positively charged," in reference to an amino acid, refers to those amino acids, amino acid derivatives, amino acid mimetics and chemical moieties that are positively charged at physiological pH. Positively charged amino acids include, for example, lysine and arginine, which are preferred positively charged amino acids for use in the invention. A "basic residue" is a residue that is positively charged at physiological pH.

The term "residue" as used herein refers to an amino acid that is incorporated into a polypeptide. The amino acid may be a naturally occurring amino acid and, unless otherwise limited, may encompass known analogs of natural amino acids that can function in a similar manner as naturally occurring amino acids.

The term "position," with respect to an amino acid residue in a polypeptide, refers to a number corresponding to the numerical place that residue holds in the polypeptide. By convention, residues are counted from the amino terminus to the carboxyl terminus of the polypeptide. Thus, position 13 of IL-13 would be the thirteenth residue from the amino terminus of the IL-13 sequence.

The term "occupy" or "occupied" with respect to a position in a polypeptide sequence refers to the amino acid residue in the particular position described. Thus, position 13 of IL-13 is usually "occupied" by a glutamic acid residue. In a mutant in which the glutamic acid is replaced by a lysine residue (typically by site-directed mutagenesis), the position would be said to be occupied by a lysine.

Mutations of an amino acid at a particular position are stated according to convention. Under the convention for stating changes in a protein, the original amino acid is listed first (in standard single letter code), followed by the position at which it occurs in the protein or polypeptide, and then by the amino acid replacing the original amino acid. Thus, the replacement of an arginine at position 112 by an aspartic acid is

designated by the notation: R112D. The term "IL-13R112D therefore designates an IL-13 protein in which the arginine which is normally found at position 112 has been mutated to aspartic acid.

A "fusion protein" refers to a polypeptide formed by the joining of two or more polypeptides through a peptide bond formed between the amino terminus of one polypeptide and the carboxyl terminus of another polypeptide. The fusion protein may be formed by the chemical coupling of the constituent polypeptides or it may be expressed as a single polypeptide from nucleic acid sequence encoding the single contiguous fusion protein. A single chain fusion protein is a fusion protein having a single contiguous polypeptide backbone.

A "chimeric molecule" is a single molecule created by joining two or more molecules that exist separately in their native state. The single, chimeric molecule has the desired functionality of all of its constituent molecules. Frequently, one of the constituent molecules of a chimeric molecule is a "targeting molecule" or "targeting moiety." The targeting molecule is a molecule such as a ligand or an antibody that specifically binds to its corresponding target, for example a receptor on a cell surface. Thus, for example, where the targeting molecule is a cytokine such as IL-13, the chimeric molecule will specifically bind (target) cells and tissues bearing an IL-13 receptor.

Another constituent of the chimeric molecule may be an "effector molecule." The effector molecule refers to a molecule (or, in the context of a chimeric molecule, the portion or "moiety") that is to be specifically transported to the target to which the chimeric molecule is specifically directed. The effector molecule typically has a characteristic activity that is desired to be delivered to the target cell. Effector molecules include cytotoxins, labels, radionuclides, ligands, antibodies, drugs, liposomes (including liposomes loaded with a drug whose delivery to the target cell is desired), and the like.

The term "specifically deliver" as used herein refers to the preferential association of a molecule with a cell or tissue bearing a particular target molecule or marker and not to cells or tissues lacking that target molecule. It is, of course, recognized that a certain degree of non-specific interaction may occur between a molecule and a non-target cell or tissue. Nevertheless, specific delivery, may be distinguished as mediated through specific recognition of the target molecule. Typically specific delivery results in a much stronger association between the delivered molecule and cells bearing the target molecule than between the delivered molecule and cells lacking the target molecule.

Specific delivery typically results in greater than 2 fold, preferably greater than 3 fold, more preferably 10 fold or greater and most preferably greater than 100 fold increase in amount of delivered molecule (per unit time) to a cell or tissue bearing the target molecule as compared to a cell or tissue lacking the target molecule or marker.

A "spacer" as used herein refers to a peptide that joins the proteins comprising a fusion protein. Generally a spacer has no specific biological activity other than to join the proteins or to preserve some minimum distance or other spatial relationship between them. However, the constituent amino acids of a spacer may be selected to influence some property of the molecule such as the folding, net charge, or hydrophobicity of the molecule.

A "ligand", as used herein, refers generally to all molecules capable of reacting with or otherwise recognizing or binding to a receptor on a target cell. Specifically, examples of ligands include, but are not limited to, antibodies, lymphokines, cytokines, receptor proteins such as CD4 and CD8, solubilized receptor proteins such as soluble CD4, hormones, growth factors, and the like which specifically bind desired target cells.

The term "cpIL-13" is used to designate a circularly permuted (cp) IL-13. Circular permutation is functionally equivalent to taking a straight-chain molecule, fusing the ends (directly or through a linker) to form a circular molecule, and then cutting the circular molecule at a different location to form a new straight chain molecule with different termini.

A "conservative substitution", when describing a protein refers to a change in the amino acid composition of the protein that does not substantially alter the protein's activity. Thus, "conservatively modified variations" of a particular amino acid sequence refers to amino acid substitutions of those amino acids that are not critical for protein activity or substitution of amino acids with other amino acids having similar properties (*e.g.*, acidic, basic, positively or negatively charged, polar or non-polar, etc.) such that the substitutions of even critical amino acids do not substantially alter activity. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such substitutions preferably are made in accordance with the following list, which substitutions may be determined by routine experimentation provide modified structural and functional properties of a synthesized polypeptide molecule, while maintaining the receptor binding, or inhibiting or mimicking biological activity, of IL-13, as determined by, for example, competitive binding, proliferation, and cytotoxicity assays.

Original Residue	Exemplary Substitution
Ala	Gly; Ser
Arg	Lys
Asn	Gln; His
Asp	Glu
Cys	Se
Gln	Asn
Glu	Asp
Gay	Ala; Pro
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Tyr; Ile
Phe	Met; Leu; Tyr
Se	Thr
Thr	Se
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Put differently, the following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q);
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

See also, Creighton (1984) PROTEINS, W.H. Freeman and Company. In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also " conservatively modified variations".

The term "substantial identity" or "substantial similarity" in the context of a polypeptide indicates that a polypeptides comprises a sequence which can have 40% sequence identity to a reference sequence, or preferably 70%, or more preferably 85% sequence identity to the reference sequence, or even more preferably 90% identity over a comparison window of about 10-20 amino acid residues.

"Percentage amino acid identity" or "percentage amino acid sequence identity" refers to a comparison of the amino acids of two polypeptides which, when optimally aligned, have approximately the designated percentage of the same amino acids. For example, "95% amino acid identity" refers to a comparison of the amino acids of two polypeptides which when optimally aligned have 95% amino acid identity. The IL-13R binding molecules of the invention have at least about 85% identity to wtIL-13, preferably about 90% identity, and more preferably about 95% identity. For convenience of reference herein, "identity" and "identical" in the context of comparing an IL-13 receptor binding molecule to wild type IL-13 in percentage terms refers to their percentage amino acid identity or percentage amino acid sequence identity.

Preferably, residue positions which are not identical (other than the specific mutations noted herein) differ by conservative amino acid substitutions. Because the substituted amino acids have similar properties, the substitutions do not change the functional properties of the polypeptides. An indication that two polypeptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a polypeptide is substantially identical to a second polypeptide, for example, where the two peptides differ only by a conservative substitution. An indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48: 443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (*see generally* Ausubel *et al., supra*).

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35: 351-360 (1987). The method used is similar to the method described by Higgins & Sharp, CABIOS 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For purposes of this invention, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

Another example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., *J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a

affinity of IL-13 can be determined by using recombinant IL-13. Recombinant IL-13 for such assays is commercially available from a number of sources (*see, e.g.* R & D Systems, Minneapolis, Minnesota, USA, and Sanofi Bio-Industries, Inc., Tervose, Pennsylvania, USA). Alternatively, a gene or a cDNA encoding IL-13 may be cloned into a plasmid or other expression vector and expressed in any of a number of expression systems according to methods well known to those of skill in the art. Methods of cloning and expressing IL-13 and the nucleic acid sequence for IL-13 are well known (see, for example, Minty *et al.* (1993) *supra,* and McKenzie *et al., supra).* In addition, the expression of IL-13 as a component of a chimeric molecule is detailed in Example 4.

One of skill in the art will appreciate that analogues or fragments of IL-13 bearing will also specifically bind to the IL-13 receptor. For example, conservative substitutions of residues (*e.g.*, a serine for an alanine or an aspartic acid for a glutamic acid) comprising native IL-13 will provide IL-13 analogues that also specifically bind to the IL-13 receptor. Thus, the term "IL-13", when used in reference to a targeting molecule, also includes fragments, analogues or peptide mimetics of IL-13 that also specifically bind to the IL-13 receptor.

B) Circularly permuted IL-13.

In another embodiment of this invention, the targeting moiety can be a circularly permuted IL-13 (cpIL-13) mutated by non-conservative changes of residues corresponding to residues 92, 110, or 112 of IL-13, or non-conservative changes to combinations of these residues. Circular permutation is functionally equivalent to taking a straight-chain molecule, fusing the ends (directly or through a linker) to form a circular molecule, and then cutting the circular molecule at a different location to form a new straight chain molecule with different termini (*see, e.g.,* Goldenberg, *et al. J. Mol. Biol.*, 165: 407-413 (1983) and Pan *et al. Gene* 125: 111-114 (1993)). Circular permutation thus has the effect of essentially preserving the sequence and identity of the amino acids of a protein while generating new termini at different locations.

Circular permutation of IL-13 provides a means by which the native IL-13 protein may be altered to produce new carboxyl and amino termini without diminishing the specificity and binding affinity of the altered first protein relative to its native form. With new termini located away from the active (binding) site, it is possible to incorporate the circularly permuted IL-13 into a fusion protein with a reduced, or no diminution, of IL-13 binding specificity and/or avidity.

It will be appreciated that while circular permutation is described in terms of linking the two ends of a protein and then cutting the circularized protein these steps are not actually required to create the end product. A protein can be synthesized *de novo* with the sequence corresponding to a circular permutation of the native protein. Thus, the

word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci.* USA 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci.* USA 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For purposes of this invention, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

The term "nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g. degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka et al., *J. Biol. Chem.* 260:2605-2608 (1985); and Cassol et al., 1992; Rossolini et al., *Mol. Cell.*

Probes 8: 91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

The phrase "a nucleic acid sequence encoding" refers to a nucleic acid which contains sequence information for a structural RNA such as rRNA, a tRNA, or the primary amino acid sequence of a specific protein or peptide, or a binding site for a trans-acting regulatory agent. This phrase specifically encompasses degenerate codons (*i.e.*, different codons which encode a single amino acid) of the native sequence or sequences which may be introduced to conform with codon preference in a specific host cell.

III. Uses of IL-13 Agonists

A. Activation of Dendritic Cells

Recent studies have demonstrated that peripheral blood derived- or bone marrow derived- dendritic cells ("DC") are very potent professional antigen presenting cells ("APCs"). These cells in clinical trials as vaccines to augment immune responses to cancer and infection by HIV. Typically, peripheral blood derived monocytes are collected from a normal donor or a cancer patient and cultured with IL-4 and granulocyte-macrophage colony stimulating factor (GM-CSF). After 6-10 days, these cells are generally found to be differentiated into dendritic cells, which show unique surface phenotypes. For example, they begin to express CD11c, CD80, and CD83, and MHC class II expression is increased. The DCs are then treated in a manner to "load" them with an antigen relevant to the condition against which it is desired to augment the immune response. Loading is usually accomplished by pulsing the cells with antigen, tumor cell lysates, peptide antigens, or apoptotic bodies, by fusing them to whole tumor cells, or by transforming the cells with genes which express the desired antigens, with or without co-stimulatory molecules. The treated cells are then washed and injected to patients at multiple time point schedules to boost the immune response. The treated DCs migrate to lymph nodes, where they express the antigen in the context of MHC class I molecules, thereby "educating" cytotoxic T cells to recognize the antigen. These T cells then circulate and kill cells that express the antigen.

The success of these forms of immune therapy depend on the generation of mature DCs. Recent studies have shown that IL-13 can replace IL-4 in the activation of DCs. As little as 10 ng/ml IL-13, when combined with 10 to 100 ng/ml GM-CSF, can generate dendritic cells from peripheral blood monocytes. Since IL-13R112D is 5 to 10 times as potent an agonist of IL-13 activity, it can be used in place of IL-13, but considerably smaller amounts need to be used to generate dendritic cells compared to the amount of IL-13 which is required. One can use as little as 0.1 ng/ml of IL-13R112D along with GM-CSF in the protocol taught in the Examples to activate DCs. Other

agonists of IL-13 activity, such as IL-13E110K, IL-13E110R, IL-13R109D, IL-13R109E, IL-13E92K, IL-13E92R, and mutants of IL-13 with a D or an E at position 66 or position 69, can be used in the same manner in the maturation and activation of potent DCs. Thus, the agonists of the invention provide a reagent for DC maturation and activation that eliminates dependence on the production of large amounts of IL-13.

B. Pretreatment of Bone Marrow Stem Cell Donors

Graft-versus-host disease (GVHD) following allogeneic stem cell transplantation is still a major obstacle to an otherwise highly useful treatment. Various studies have demonstrated that type-1 T lymphocytes (secreting interleukin ("IL")-2 and interferon-gamma) in harvested donor cells mediate acute GVHD, whereas type-2 T lymphocytes (secreting IL-4 and IL-10) can prevent acute GVHD. Type-2 T cells also produce IL-13.

Pan et al., Blood 86:4422-4429 (1995), reported that, in an animal model, pretreatment of donors with granulocyte colony-stimulating factor (G-CSF) suppressed the severity and incidence of acute GVHD, despite the fact that large numbers of lymphocytes remained in the donor infusion. It was noted that type 1 T-cells (which secrete IL-2 and interferon gamma) mediate acute GVHD, while type 2 T-cells, which secrete IL-4 and IL-10, can prevent GVHD. Pretreatment of donors with G-CSF polarize donor T lymphocytes toward type-2 cytokine production, which is associated with reduced type 1 cytokine production and reduced severity of experimental GVHD. *Id.*

Wild type IL-13 polarizes T-cells towards type-2 cytokine production and can be used along with G-CSF to decrease the severity of GVHD. Given the greater potency of the agonists of the invention, it is expected that the agonists can be used in place of wtIL-13 at the same dose with greater effect or in smaller amounts to achieve the same effect. Typically. donors will be treated on an alternate day schedule with IL-13R112D (doses between 1 to 100 microgram/Kg body weight) for two weeks prior to the harvesting of bone marrow cells. The cells collected be tested for type 2 polarization by determining their cytokine output. Cells with high levels of type 2 cytokines relative to type 1 cytokine production will be infused into the recipient. Suitable recipients include those in need of transplantation for cancer therapy, or in need of rescue of marrow after intensive chemotherapy.

Agonists of IL-13 activity suitable for use in the polarization of T-cells to decrease GVHD include IL-13E110K, IL-13E110R, IL-13R109D, IL-13R109E, IL-13E92K, IL-13E92R, and mutants of IL-13 with a D or an E at position 66 or position 69. IL-13R112D is the most preferred agonist for this use.

IV. Use of IL-13 Antagonists in Asthma, Allergic Rhinitis, Atopic Dermatitis, Cancer, and Other Conditions

A. Asthma

IL-13 is a necessary and sufficient factor for the expression of allergic asthma. IL-13 induces pathophysiological features of asthma in animals in a manner that is independent of IgE, IL-4, and eosinophils and is now considered the central mediator of allergic asthma. See, e.g., Wills-Karp, M., et al., Science 282:2258-22621 (1998). Further studies in patients with asthma have demonstrated that IL-13 is locally produced in the bronchoalveolar lavage (BAL) cells when challenged with allergen (Huang, et al. J. Immunol. 155:2688, (1995)). Up to 3 ng/ml of IL-13 protein/ml BAL was detected in allergen challenged patients.

As noted by Van der Pouw Kraan, T., et al., Clin Exp Immunol 111:129-135 (1998), IgE antibodies play a crucial role in allergic type 1 reactions. A study of the role of IL-13 in IgE synthesis in allergic asthma patients showed IgE production in allergic asthma patients is more dependent on IL-13 than in non-patients due to enhanced IL-13 production and to enhanced IgE production in response to IL-13.

These results indicate that neutralization of endogenous IL-13 will diminish the production of IgE and, therefore, the onset or strength of asthmatic attacks. Since IL-13 induces pathophysiological features of asthma in animals in a manner that is independent of IgE, IL-4, and eosinophils, IL-13 antagonists are helpful in both IgE dependent and in IgE-independent asthma. The antagonists of IL-13 of the invention are expected to neutralize the effect of IL-13 and thus will be useful for preventing the onset of asthma, or for decreasing the severity of asthma once it has developed.

Since local production of IL-13 is important in the pathophysiology of asthma, the intranasal route is preferred for therapeutic applications. This route of administration can be easily performed by patients, as many patients are already familiar with the use of inhalers used in connection with other drugs for the treatment of asthma. Typically, the amount of wt IL-13 in bronchoalveolar lavage (BAL) cells is determined (for example, up to 3 ng/ml BAL) in patients with allergen induced asthma, and a ten fold higher amount of antagonist of the invention is administered. Typical intranasal doses are 30 ng/ml to 30 microgram/ml, administered every alternate day for two weeks. The antagonists can also be administered by systemic i.v. administration, 1 to 50 microgram/Kg doses every alternate day for two weeks. In severe cases, or where immediate suppression of the asthma is required, a continuous infusion can be administered, using higher doses (100 micrograms/kg) of antagonist.

B. Allergies, including Allergic Rhinitis

Studies in an animal model with a mutated IL-4 which is also an antagonist for IL-13 have indicated that the antagonist inhibited allergic responses and allergic symptoms mediated either by IgE or IgG1. E.g., Grunewald, S. et al., J. Immunol 160:4004-4009 (1998). Because IL-4 inhibitors not only block the effect of IL-4 but also the effect of IL-13 through shared receptors on some cell types, IL-4 mutants can also block the effect of IL-13 in certain systems. However, since the IL-13Rα chain is not shared with IL-4R, and since this chain binds IL-13 with strong affinity, IL-4 mutants do not block the effect of IL-13 on every cell type. The antagonists of the invention are therefore more specific than the IL-4/IL-13 antagonists used by Grunewald et al., and are expected to inhibit allergic responses and symptoms more completely than did the mutated IL-4 antagonist used in the Grunewald et al. study. Accordingly, the IL-13 antagonists of the invention, such as IL-13E13K and IL-13E13KR112D, are particularly useful for reducing or eliminating allergic responses.

C. Atopic Dermatitis

The synthesis of IgE is an important factor in the development and maintenance of atopic dermatitis in patients. It has been reported that levels of mRNA for IL-13 were significantly higher in the peripheral blood mononuclear cells (PBMC) of patients with atopic dermatitis than in PBMC of controls. See, Katagiri, K. et al., Clin Exp Immunol 108:298-294 (1997). As noted in the subsection on asthma, above, IL-13 is implicated in the synthesis of IgE, and IL-13 antagonists can diminish the synthesis of IgE. Accordingly, the antagonists of the invention are expected to diminish the symptoms and longevity of atopic dermatitis.

D. Inflammatory conditions

IL-13 has been shown to be involved in inflammatory conditions in addition to those listed above. Specifically, IL-13 is implicated in hepatic fibrosis induced by schistosomiasis and in susceptibility to Leishmania major infection. Administration of IL-13 antagonists ameliorates the formation of hepatic fibrosis and reduces susceptibility to infection by Leishmania major.

E. Cancers

As noted in some detail in Section I E, above, IL-13 has been shown to function as an autocrine factor in Hodgkin's disease. See, e.g., Kapp et al., J. Exp. Med. 189:1939-1945 (1999). Neutralizing antibodies to IL-13 were shown to block the

proliferation of these cells in a dose dependent manner. *Id.* Additionally, Kaposi's sarcoma cells and renal cancer cells have been shown to secrete IL-13, and antibodies to IL-13 have been shown to inhibit the growth of Kaposi's sarcoma cells, showing that IL-13 has an autocrine effect on these cells as well. Accordingly, the antagonists of the invention can be used to slow the growth of HD/RS cells, Kaposi's sarcoma cells, and renal cell carcinoma cells.

The antagonists of the invention can further be used to slow or to stop the growth of other cancers in which IL-13 increases proliferation or acts as an autocrine growth factor. Such cancers can be readily identified. For example, Kapp et al., *supra*, describe the confirmation of IL-13 expression by tumor cell lines using Northern blots and ELISA assays, as well as assays for showing that an antagonist blocks proliferation of such cells in a dose dependent manner. Additional assays are described in Examples 19 and 20, below. In a typical protocol, the cells are washed to remove any endogenously-secreted IL-13 (to provide a baseline), and divided into cultures. IL-13 is added to all but one of the cultures (the one to which IL-13 is not added remains as a control), one culture is left with only IL-13 added, and an IL-13 antagonist is added to the other cultures, with each succeeding culture receiving an increasing amount of antagonist. The growth of cancers in which IL-13 is a growth factor will be inhibited by the antagonist in a dose-dependent manner, while the growth of cancers in which IL-13 is not a growth factor is not affected.

It should be noted that in addition to the cancers mentioned above, a number of cancers, including gliomas, medulloblastomas, about 25% of head and neck cancers, and some pancreatic cancers, overexpress the IL-13 receptor. It is expected that these cancers proliferate in the presence of IL-13 and that contacting cells of these cancers with the antagonists of the invention will stop or slow the proliferation of the cells.

V. Chimeric Molecules Targeted to the IL-13 Receptor.

The present invention provides compositions, including molecules with higher binding affinity for IL-13R than that of native IL-13, chimeric molecules which use these higher affinity molecules as targeting agents coupled to an effector molecule, and methods for specifically delivering an effector molecule to a tumor cell. The methods involve the use of chimeric molecules comprising a targeting molecule attached

to an effector molecule. The chimeric molecules of this invention permit delivery of effector molecules which specifically target tumor cells.

The improved specific targeting of this invention is premised, in part, on previous findings from our laboratory that solid tumors, especially carcinomas, express IL-13 receptors at extremely high levels compared to normal tissues. For example, while the IL-13 receptors (IL-13R) are overexpressed on tumor cells, expression on other cells (*e.g.* monocytes, B cells, and T cells) appears negligible. Thus, by specifically targeting the IL-13 receptor, the present invention provides chimeric molecules that are specifically directed to solid tumors while minimizing targeting of other cells or tissues.

In a preferred embodiment, this invention provides for compositions and methods for impairing the growth of tumors. These methods involve providing a chimeric molecule comprising a cytotoxic effector molecule attached to a targeting molecule which binds to IL-13R with an affinity at least about three times that of wild type IL-13. The cytotoxic effector molecule may be a native or modified cytotoxin such as *Pseudomonas* exotoxin (PE), *Diphtheria* toxin (DT), ricin, abrin, saponin, gelonin, ribosome inactivating protein, and the like, or it may be a radionuclide, a drug, or a liposome which is itself loaded with a drug or other cytotoxic agent. In a preferred embodiment, the cytotoxic molecule (or "moiety") is a cytotoxin. In even more preferred embodiments, the cytotoxin is a peptide or protein, which can be expressed as a single chain fusion protein with the targeting molecule.

The chimeric cytotoxin is administered to an organism containing tumor cells which are then contacted by the chimeric molecule. The targeting molecule component of the chimeric molecule specifically binds to the overexpressed IL-13 receptors on the tumor cells. Once bound to the IL-13 receptor on the cell surface, the cytotoxic effector molecule mediates internalization into the cell where the cytotoxic molecule inhibits cellular growth or kills the cell.

The use of chimeric molecules comprising a targeting moiety joined to a cytotoxic effector molecules to target and kill tumor cells is known in the prior art. For example, chimeric fusion proteins which include interleukin 4 (IL-4) or transforming growth factor (TGFα) fused to *Pseudomonas* exotoxin (PE) or interleukin 2 (IL-2) fused to *Diphtheria* toxin (DT) have been tested for their ability to specifically target and kill cancer cells (Pastan *et al., Ann. Rev. Biochem.*, 61: 331-354 (1992)).

Prior work from our laboratory showed that although chimeric IL-4-cytotoxin molecules are known in the prior art, and IL-4 shows some sequence similarity to IL-13, cytotoxins targeted by a moiety specific to the IL-13 receptor had significantly increased efficacy as compared to IL-4 receptor directed cytotoxins. As noted in U.S. Patent No. 5,919,456 and 5,614,191, this appears to be due to at least three factors.

First, IL-13 receptors are expressed at much lower levels, if at all on non-tumor cells (*e.g.* monocytes, T cells, B cells). Thus cytotoxins directed to IL-13 receptors show reduced binding and subsequent killing of healthy cells and tissues as compared to other cytotoxins.

Second, the receptor component that specifically binds IL-13 appears to be expressed at significantly higher levels on solid tumors than the receptor component that binds IL-4. Thus, tumor cells bind higher levels of cytotoxic chimeric molecules directed against IL-13 receptors than cytotoxic chimeric molecules directed against IL-4 receptors.

Third, and finally, IL-4 receptors are up-regulated when immune system cells (*e.g.* T-cells) are activated. This results in healthy cells, for example T-cells and B-cells, showing greater susceptibility to IL-4 receptor directed cytotoxins. Thus, the induction of an immune response (as against a cancer), results in greater susceptibility of cells of the immune system to the therapeutic agent. In contrast, IL-13 receptors have not been shown to be up-regulated in activated T cells. Thus IL-13 receptor targeted cytotoxins have no greater effect on activated T cells and thereby minimize adverse effects of the therapeutic composition on cells of the immune system.

In another embodiment, this invention also provides for compositions and methods for detecting the presence or absence of tumor cells. These methods involve providing a chimeric molecule comprising an effector molecule, that is a detectable label attached to a targeting molecule of the invention. The IL-13 receptor targeting moiety specifically binds the chimeric molecule to tumor cells with high affinity. The cells are then marked by their association with the detectable label. Subsequent detection of the cell-associated label indicates the presence of a tumor cell.

In yet another embodiment, the effector molecule may be another specific binding moiety such as an antibody, a growth factor, or a ligand. The chimeric molecule will then act as a highly specific bifunctional linker. This linker may act to bind and enhance the interaction between cells or cellular components to which the chimeric molecule binds. Thus, for example, where the "targeting" component of the chimeric molecule comprises a polypeptide of the invention that specifically binds to an IL-13 receptor and the "effector" component is an antibody or antibody fragment (*e.g.* an Fv fragment of an antibody), the targeting component specifically binds cancer cells, while the effector component binds receptors (*e.g.,* IL-2 or IL-4 receptors) on the surface of immune cells. The chimeric molecule may thus act to enhance and direct an immune response toward target cancer cells.

In still yet another embodiment the effector molecule may be a pharmacological agent (*e.g.* a drug) or a vehicle containing a pharmacological agent. This is particularly suitable where it is merely desired to invoke a non-lethal biological

response. Thus the moiety that specifically binds to an IL-13 receptor may be conjugated to a drug such as vinblastine, doxirubicin, genistein (a tyrosine kinase inhibitor), an antisense molecule, or other pharmacological agents known to those of skill in the art, thereby specifically targeting the pharmacological agent to tumor cells over expressing IL-13 receptors.

Alternatively, the targeting molecule may be bound to a vehicle containing the therapeutic composition. Such vehicles include, but are not limited to liposomes, micelles, various synthetic beads, and the like.

One of skill in the art will appreciate that the chimeric molecules of the present invention may include multiple targeting moieties bound to a single effector or conversely, multiple effector molecules bound to a single targeting moiety. In still other embodiment, the chimeric molecules may include both multiple targeting moieties and multiple effector molecules. Thus, for example, this invention provides for "dual targeted" cytotoxic chimeric molecules in which an IL-13 receptor binding molecule of the invention is attached to a cytotoxic molecule and another molecule (*e.g.* an antibody, or another ligand) is attached to the other terminus of the toxin. Such a dual-targeted cytotoxin might comprise a PE substituted for domain Ia at the amino terminus of a PE and anti-TAC(Fv) inserted in domain III, between amino acid 604 and 609. Other antibodies may also be suitable.

VI. The Targeting Molecule.

In a preferred embodiment, the targeting molecule is a molecule that specifically binds to the IL-13 receptor with at least about 3 times the affinity of wild type IL-13. In preferred embodiments, the molecule binds to the IL-13 receptor with an affinity at least about 5 times the affinity of wild type IL-13 and, in more preferred embodiments, the molecules bind with at least about 10 times the affinity or more. The term "specifically binds", as used herein, when referring to a protein or polypeptide, refers to a binding reaction which is determinative of the presence of the protein or polypeptide in a heterogeneous population of proteins and other biologics. Thus, under designated conditions, the specified molecule binds to its particular "target" protein (*e.g.* an IL-13 receptor) and does not bind in a significant amount to other proteins present in the sample or to other proteins to which the ligand may come in contact in an organism.

Assay formats for detecting specific binding of ligands (*e.g.* an IL-13 receptor binding molecule) with their respective receptors are well known in the art. The Examples provide a protocol for assessing the competitive binding of IL-13 receptor binding molecules of the invention compared to wild type IL-13.

The IL-13 receptor is a cell surface receptor that specifically binds IL-13 and mediates a variety of physiological responses in various cell types as described below

in the description of IL-13. The IL-13 receptor may be identified by contacting a cell or other sample with labeled IL-13 and detecting the amount of specific binding of IL-13 according to methods well known to those of skill in the art.

Alternatively, an anti-IL-13 receptor antibody may also be used to identify IL-13 receptors. The antibody will specifically bind to the IL-13 receptor and this binding may be detected either through detection of a conjugated label or through detection of a labeled second antibody that binds the anti-IL-13 receptor antibody.

In a preferred embodiment, the moiety utilized to specifically target the IL-13 receptor is an IL-13 receptor binding molecule with at least about 3 times the binding affinity for the IL-13 receptor of wild type IL-13. In particularly preferred embodiments, the moiety is an IL-13 or cpIL-13 mutated as taught herein and that specifically binds the IL-13 receptor with an affinity at least about 3 times that of wild type IL-13.

A) IL-13

As noted above, interleukin-13 (IL-13) is a pleiotropic cytokine that is recognized to share many of the properties of IL-4, with which it shares approximately 30% sequence identity. It exhibits IL-4-like activities on monocytes/macrophages and human B cells (Minty *et al., Nature*, 362: 248 (1993), McKenzie *et al. Proc. Natl. Acad. Sci. USA*, 90:3735-3739 (1987) ("McKenzie *et al.*").

The nucleotide and amino acid sequences of human IL-13 were determined and set forth in the publication by McKenzie *et al., supra*, and are also available on the Internet at, for example, the Entrez browser of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) under accession number L06801. The first eighteen (18) amino acid residues of the sequence set forth by McKenzie *et al.* (through and including the third alanine) are considered in the art to be a signal sequence and the mature IL-13 protein is considered to commence with the nineteenth residue, a serine. SEQ ID NO:1 sets forth the translation (including both the signal sequence and the mature IL-13 sequence) as deposited by McKenzie *et al.*, in GenBank under the accession number noted above. Figure 1 shows the sequence of mature human IL-13 and compares it to the sequences of mature IL-13 of other species. References herein to particular residues of IL-13, such as residues 92, 110, and 112, and to percentages of identity to IL-13, are to the amino acid sequence of mature human IL-13. The sequence of mature human IL-13 set forth in Figure 1 (and derivable from McKenzie *et al.* by commencing with the serine residue noted above) is also referred to herein as "native" or "wild type" IL-13. SEQ ID NO:2 is the nucleotide sequence for human IL-13, including the signal sequence and non-coding regions.

The IL-13R binding molecules of the invention have at least about 3 times the binding affinity for the IL-13R than does wild type IL-13. Conveniently, the binding

term "circularly permuted IL-13 (cpIL-13)" refers to all IL-13 proteins having a sequence corresponding to a circular permutation of a native IL-13 protein regardless of how they are constructed.

Generally, a permutation that retains or improves the binding specificity and/or avidity (as compared to the native IL-13) is preferred. If the new termini interrupt a critical region of the native protein, binding specificity and avidity may be lost. Similarly, if linking the original termini destroys IL-13 binding specificity and avidity then no circular permutation is suitable. Thus, there are two requirements for the creation of an active circularly permuted protein: 1) the termini in the native protein must be favorably located so that creation of a linkage does not destroy binding specificity and/or avidity; and 2) there must exist an "opening site" where new termini can be formed without disrupting a region critical for protein folding and desired binding activity (*see, e.g.,* Thorton *et al. J. Mol. Biol.,* 167: 443-460 (1983)). This invention establishes that IL-13 meets these criteria and provides for circularly permuted IL-13 that having improved binding characteristics.

When circularly permuting IL-13, it is desirable to use a linker that preserves the spacing between the termini comparable to the unpermuted or native molecule. Generally linkers are either hetero- or homo-bifunctional molecules that contain two reactive sites that may each form a covalent bond with the carboxyl and the amino terminal amino acids respectively. Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide linkers. The most common and simple example is a peptide linker that typically consists of several amino acids joined through peptide bonds to the termini of the native protein. The linkers may be joined to the terminal amino acids through their side groups (*e.g.*, through a disulfide linkage to cysteine). In preferred embodiments, however, the linkers will be joined to the alpha carbon amino and carboxyl groups of the terminal amino acids.

Functional groups capable of forming covalent bonds with the amino and carboxyl terminal amino acids are well known to those of skill in the art. For example, functional groups capable of binding the terminal amino group include anhydrides, carbodimides, acid chlorides, activated esters and the like. Similarly, functional groups capable of forming covalent linkages with the terminal carboxyl include amines, alcohols, and the like. In a preferred embodiment, the linker will itself be a peptide and will be joined to the protein termini by peptide bonds. A preferred linker for the circular permutation of IL-13 is Gly-Gly-Ser-Gly.

In a preferred embodiment, circular permutation of IL-13 involves creating an opening such that the formation of new termini does not interrupt secondary structure crucial to the formation of a structure that specifically binds the IL-13 receptor. Even if

the three-dimensional structure is compatible with joining the termini, it is conceivable that the kinetics and thermodynamics of folding would be greatly altered by circular permutation if the cleavage separates residues that participate in short range interactions that are crucial for the folding mechanism or the stability of the native state. Goldenberg, *Protein Eng.*, 7: 493-495 (1989). Thus, the choice of a cleavage site can be important to the protein's binding specificity and/or avidity.

The selection of an opening site in IL-13 may be determined by a number of factors. Preferred opening sites will be located in regions that do not show a highly regular three-dimensional structure. Thus, it is preferred that cleavage sites be selected in regions of the protein that do not show secondary structure such as alpha helices, pleated sheets, αß barrel structures, and the like.

Methods of identifying regions of particular secondary structure of IL-13 based on amino acid sequence are widely known to those of skill in the art. See, for example, Cohen *et al., Science,* 263: 488-489 (1994). Numerous programs exist that predict protein folding based on sequence data. Some of the more widely known software packages include MatchMaker (Tripos Associates, St. Louis, Missouri, USA), FASMAN from GCG (Genetics Computer Group), PHD (European Molecular Biology Laboratory, Heidelburg, Germany) and the like. In addition, the amino acid sequence of IL-13 is well known and the protein has been extensively characterized (*see, e.g.,* WO 94/04680).

Alternatively, where the substitution of certain amino acids or the modification of the side chains of certain amino acids does not change the activity of a protein, it is expected that the modified amino acids are not critical to the protein's activity. Thus, amino acids that are either known to be susceptible to modification or are actually modified *in vivo* are potentially good candidates for cleavage sites.

Where the protein is a member of a family of related proteins, one may infer that the highly conserved sequences are critical for biological activity, while the variable regions are not. Preferred cleavage sites are then selected in regions of the protein that do not show highly conserved sequence identity between various members of the protein family. Alternatively, if a cleavage site is identified in a conserved region of a protein, that same region provides a good candidate for cleavage sites in a homologous protein.

Methods of determining sequence identity are well known to those of skill in the art. Sequence comparisons between two (or more) polynucleotides or polypeptides are typically performed by comparing sequences of the two sequences over a "comparison window" to identify and compare local regions of sequence similarity. Since the goal is to identify very local sequence regions that are not conserved, the comparison window will be selected to be rather small. A "comparison window", as used herein, refers to a

segment of at least about 5 contiguous positions, usually about 10 to about 50, more usually about 15 to about 40 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith *et al. Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman *et al., J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson *et al., Proc. Natl. Acad. Sci. USA*, 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection.

A preferred opening site in IL-13 is just prior to Met-44 of hIL-13, just at the beginning of the putative second alpha-helix resulting in a circularly permuted IL-13 having a methionine at position 44 of the native IL-13 at the amino terminus of the cpIL-13 and the glycine at position 43 of the native IL-13 at the new carboxyl terminus of the cpIL-13. This carboxyl terminus can be joined to a second protein directly or though a spacer.

Circularly permuted IL-13 may be made by a number of means known to those of skill in the art. These include chemical synthesis, modification of existing proteins, and expression of circularly permuted proteins using recombinant DNA methodology.

The circularly permuted IL-13 may be synthesized using standard chemical peptide synthesis techniques as discussed below. If the linker is a peptide it may be incorporated during the synthesis. If the linker is not a peptide it may be coupled to the peptide after synthesis.

Alternatively, the circularly permuted IL-13 can be made by chemically modifying a native IL-13 (*e.g.* a native human IL-13). Generally, this requires reacting the IL-13 in the presence of the linker to form covalent bonds between the linker and the carboxyl and amino termini of the protein, thus forming a circular protein. New termini are then formed by cleaving the peptide bond joining amino acids at another location. This may be accomplished chemically or enzymatically using, for example, a peptidase.

If the cleavage reaction tends to hydrolyze more than one peptide bond, the reaction may be run briefly. Those molecules having more than one peptide bond cleaved will be shorter than the full length circularly permuted molecule and the latter may be isolated by any protein purification technique that selects by size (*e.g.*, by size exclusion chromatography or electrophoresis). Alternatively, various sites in the circular protein may be protected from hydrolysis by chemical modification of the amino acid

side chains which may interfere with enzyme binding, or by chemical blocking of the vulnerable groups participating in the peptide bond.

In a preferred embodiment, the circularly permuted IL-13, or fusion proteins comprising the circularly permuted IL-13 is synthesized using recombinant DNA methodology. Generally this involves creating a DNA sequence that encodes the circularly permuted IL-13 (or entire fusion protein containing the IL-13), placing the DNA in an expression cassette under the control of a particular promoter, expressing the protein in a host, isolating the expressed protein and, if required, renaturing the protein. Recombinant expression of the fusion proteins of this invention is discussed in more detail below.

DNA encoding circularly permuted IL-13 or fusion proteins comprising circularly permuted IL-13 may be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences or direct chemical synthesis by methods as discussed below. Alternatively, subsequences may be cloned and the appropriate subsequences cleaved using appropriate restriction enzymes. The fragments may then be ligated to produce the desired DNA sequence.

In a preferred embodiment, DNA encoding the circularly permuted IL-13 may be produced using DNA amplification methods, for example polymerase chain reaction (PCR). First, the segments of the native DNA on either side of the new terminus are amplified separately. The 5' end of the one amplified sequence encodes the peptide linker, while the 3' end of the other amplified sequence also encodes the peptide linker. Since the 5' end of the first fragment is complementary to the 3' end of the second fragment, the two fragments (after partial purification, *e.g.* on LMP agarose) can be used as an overlapping template in a third PCR reaction. The amplified sequence will contain codons the segment on the carboxy side of the opening site (now forming the amino sequence), the linker, and the sequence on the amino side of the opening site (now forming the carboxyl sequence). The circularly permuted molecule may then be ligated into a plasmid and expressed as discussed below.

C. Modified IL-13

One of skill in the art will appreciate that IL-13 can be modified in a variety of ways that do not destroy binding specificity and/or avidity and, in fact, may increase binding properties. Some modifications may be made to facilitate the cloning, expression, or incorporation of the circularly permuted growth factor into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids placed on either terminus to create conveniently located restriction sites or termination codons.

One of skill will recognize that other modifications may be made. Thus, for example, amino acid substitutions may be made that increase specificity or binding affinity of the circularly permuted protein, *etc.* Alternatively, non-essential regions of the molecule may be shortened or eliminated entirely. Thus, where there are regions of the molecule that are not themselves involved in the activity of the molecule, they may be eliminated or replaced with shorter segments that merely serve to maintain the correct spatial relationships between the active components of the molecule.

D. Derivatized Peptides and Peptidomimetics

The design of chemically modified peptides and peptide mimics which are resistant to degradation by proteolytic enzymes or have improved solubility or binding properties is well known.

Modified amino acids may contain additional chemical moieties or modified amino acids not normally a part of a protein. Covalent modifications of the peptide are thus included within the scope of the present invention. Such modifications may be introduced into an IL-13 receptor binding molecule by reacting targeted amino acid residues of the molecule with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. The following examples of chemical derivatives are provided by way of illustration and not by way of limitation. It should be noted that to determine whether any derivatized or modified molecule is an IL-13 receptor binding molecule of the present invention, the molecule can be tested to determine whether it binds to an IL-13R with an affinity at least about 3 times that of wild type IL-13, more preferably about 5 times that of wild type IL-13, and even more preferably at least about 10 times that of wild type IL-13, or more.

The design of peptide mimics which are resistant to degradation by proteolytic enzymes is well known, both for hormone agonist/antagonist and for enzyme inhibitor design. *See e.g.*, Sawyer, in STRUCTURE-BASED DRUG DESIGN, P. Verapandia, Ed., NY 1997; U.S. Patent No. 5,552,534; and U.S. Patent No. 5,550,251, all of which are incorporated by reference.

Historically, the major focus of peptidomimetic design has evolved from receptor-targeted drug discovery research and has not been directly impacted by an experimentally-determined three-dimensional structure of the target protein. Nevertheless, a hierarchical approach of peptide→peptidomimetic molecular design and chemical modification has evolved over the past two decades, based on systematic

transformation of a peptide ligand and iterative analysis of the structure-activity and structure-conformation relationships of "second generation" analogs. Such work has typically integrated biophysical techniques (x-ray crystallography and/or NMR spectroscopy) and computer-assisted molecular modeling with biological testing to advance peptidomimetic drug design.

The three-dimensional structural properties of peptides are defined in terms of torsion angles (Ψ, φ, ω, χ) between the backbone amine nitrogen (N^{α}), backbone carbonyl carbon (C^{1}), backbone methionine carbon (C^{α}), and side chain hydrocarbon functionalization (e.g., C^{β}, C^{λ}, C^{δ}, C^{ε} of Lys) derived from the amino acid sequence. A Ramachandran plot (Ψ versus φ) may define the preferred combinations of torsion angles for ordered secondary structures (conformations), such as $^{\alpha}$ helix, $^{\beta}$ turn, $^{\gamma}$ turn, or $^{\beta}$ sheet. Molecular flexibility is directly related to covalent and/or noncovalent bonding interactions within a particular peptide. Even modest chemical modifications by N^{α}-methyl, C^{α}-methyl or C^{β}-methyl can have significant consequences on the resultant conformation.

The N^{α}-C^{α}-C′ scaffold may be transformed by introduction of olefin substitution (e.g., C^{α}-C^{β} → C = C or dehydroamino acid or insertion (e.g., C^{α}-C′ → C^{α}-C=C-C′ or vinylogous amino acid. Also the C^{β} carbon may be substituted to advance the design of so-called "chimeric" amino acids. Finally, with respect to N-substituted amides it is also noteworthy to mention the intriguing approach of replacing the traditional peptide scaffold by achiral N-substituted glycine building blocks. Overall, such N^{α}-C^{α}-C scaffold or C^{α}-C^{β} side chain modifications expand peptide-based molecular diversity (i.e., so-called "peptoid" libraries) as well as extend our 3-D structural knowledge of traditional φ-Ψ-χ space.

In one approach, such as disclosed by Sherman and Spatola, *J. Am. Chem. Soc.* 112: 433 (1990), one or more amide bonds are replaced in an essentially isosteric manner by a variety of chemical functional groups. For example, any amide linkage in an IL-13 receptor binding molecule can be replaced by a ketomethylene moiety, e.g. (-C(=O)-CH_2-) for (-(C=O)-NH-). A few of the known amide bond replacements include: aminomethylene or $\Psi[CH_2NH]$; ketomethylene or $\Psi[COCH_2]$; ethylene or $\Psi[CH_2CH_2]$; olefin or Ψ[CH=CH]; ether or $\Psi[CH_2O]$; thioether or $\Psi[CH_2S]$; tetrazole or $\Psi[CN_4]$; thiazole or Ψ[thz]; retroamide or Ψ[NHCO]; thioamide or Ψ[CSNH]; and ester or $\Psi[CO_2]$. These amide bond surrogates alter conformational and H-bonding properties that may be requisite for peptide molecular recognition and/or biological activity at

receptor targets. Furthermore, such backbone replacements can impart metabolic stability towards peptidase cleavage relative to the parent peptide. The discovery of yet other nonhydrolyzable amide bond isostere has particularly impacted the design of protease inhibitors, and these include: hydroxymethylene or Ψ[CH(OH)]; hydroxyethylene or Ψ[CH(OH)CH_2] and Ψ[CH_2CH(OH)]; dihydroxyethylene or (Ψ[CH(OH)CH(OH)], hydroxyethylamine or Ψ[CH(OH)CH_2N], dihydroxyethylene and C_2-symmetric hydroxymethylene. Such backbone modifications have been extremely effective, as they may represent transition state mimics or bioisosteres of the hypothetical tetrahedral intermediate (e.g., Ψ[$C(OH)_2$NH]) for this class of proteolytic enzymes. Such derivatives are expected to have the property of increased stability to degradation by enzymes, and therefore possess advantages for the formulation of compounds which may have increased *in vivo* half lives, as administered by oral, intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes.

Both peptide backbone and side chain modifications may provide prototypic leads for the design of secondary structure mimicry, as typically suggested by the fact that substitution of D-amino acids, N^{α}-Me-amino acids, C_{α}-Me amino acids, and/or dehydroamino acids within a peptide lead may induce or stabilize regiospecific $^{\beta}$-turn, $^{\gamma}$-turn, $^{\beta}$-sheet, or $^{\alpha}$-helix conformations. To date, a variety of secondary structure mimetics have been designed and incorporated in peptides or peptidomimetics. The $^{\beta}$-turn has been of particular interest to the area of receptor-targeted peptidomimetic drug discovery. This secondary structural motif exists within a tetrapeptide sequence in which the first and fourth C^{α} atoms are ≤ 7 Å separated, and they are further characterized as to occur in a nonhelical region of the peptide sequence and to possess a ten-membered intramolecular H-bond between the i and i⟶4 amino acid residues. One of the initial approaches of significance to the design of $^{\beta}$-turn mimetics was the monocyclic dipeptide-based template which employs side chain to backbone constraint at the i+1 and i+2 sites. Over the past decade a variety of other monocyclic or bicyclic templates have been developed as $^{\beta}$-turn mimetics. Monocyclic $^{\beta}$-turn mimetic has been described that illustrate the potential opportunity to design scaffolds that may incorporate each of the side chains (i, i+1, i+2 and i+3 positions), as well as five of the eight NH or C=O functionalities, within the parent tetrapeptide sequence, tetrapeptide sequence modeled in type I-IV $^{\beta}$-turn conformations. Similarly, a benzodiazepine template has shown utility as a $^{\beta}$-turn mimetic scaffold which also may be multisubstituted to simulate side chain functionalization,; particularly at the i and i+3 positions of the corresponding tetrapeptide

sequence modeled in type I-VI $^{\beta}$-turn conformations. A recently reported γ-turn mimetic, illustrates an innovative approach to incorporate a retroamide surrogate between the i and i→1 amino acid residues with an ethylene bridge between the N^{l} (i.e., nitrogen replacing the carbonyl C′) and N atoms of the i and i+2 positions, and this template allows the possibility for all three side chains of the parent tripeptide sequence. Finally, the design of a $^{\beta}$-sheet mimetic provides an attractive template to constrain the backbone of a peptide to that simulating an extended conformation. The $^{\beta}$-sheet is of particular interest to the area of protease-targeted peptidomimetic drug discovery.

Aromatic amino acids may be replaced with D- or L-napthylalanine, D- or L-phenylglycine, D- or L-2-thieneylalanine, D- or L-1-, 2-, 3- or 4-pyreneylalanine, D- or L-3-thieneylalanine, D- or L-(2-pyridinyl)-alanine, D- or L-(3-pyridinyl)-alanine, D- or L-(2-pyrazinyl)-alanine, D- or L-(4-isopropyl)-phenylglycine, D-(trifluoromethyl)-phenylglycine, D-(trifluoromethyl)-phenylalanine, D-p-fluorophenylalanine, D- or L-p-biphenylphenylalanine, D- or L-p-methoxybiphenylphenylalanine, D- or L-2-indole(alkyl)alanines, and D- or L-alkylainines where alkyl may be substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, isopropyl, iso-butyl, sec-isotyl, iso-pentyl, non-acidic amino acids, of C1-C20.

Acidic amino acids can be substituted with non-carboxylate amino acids while maintaining a negative charge, and derivatives or analogs thereof, such as the non-limiting examples of (phosphono)alanine, glycine, leucine, isoleucine, threonine, or serine; or sulfated (e.g., $-SO_3H$) threonine, serine, tyrosine.

Other substitutions may include unnatural hyroxylated amino acids made by combining "alkyl" (as defined and exemplified herein) with any natural amino acid. Basic amino acids may be substituted with alkyl groups at any position of the naturally occurring amino acids lysine, arginine, ornithine, citrulline, or (guanidino)-acetic acid, or other (guanidino)alkyl-acetic acids, where "alkyl" is define as above. Nitrile derivatives (e.g., containing the CN-moiety in place of COOH) may also be substituted for asparagine or glutamine, and methionine sulfoxide may be substituted for methionine. Methods of preparation of such peptide derivatives are well known to one skilled in the art.

In addition, any amino acid can be replaced by the same amino acid but of the opposite chirality. Thus, any amino acid naturally occurring in the L-configuration (which may also be referred to as the R or S, depending upon the structure of the chemical entity) may be replaced with an amino acid of the same chemical structural

type, but of the opposite caroled, generally referred to as the D- amino acid but which can additionally be referred to as the R- or the S-, depending upon its composition and chemical configuration. Such derivatives have the property of greatly increased stability co degradation by enzymes, and therefore are advantageous in the formulation of compounds which may have longer *in vivo* half lives, when administered by oral, intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes.

Additional amino acid modifications may include the following: Cysteinyl residues may be reacted with alpha-haloacetates (and corresponding amines), such as 2-chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues may also be derivatized by reaction with compounds such as bromotrifluoroacetone, alpha-bromo-beta-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues may be derivatized by reaction with compounds such as diethylprocarbonate e.g., at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain, and para-bromophenacyl bromide may also be used; e.g., where the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues may be reacted with compounds such as succinic or other carboxylic acid anhydrides. Derivatization with these agents is expected to have the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino-containing residues include compounds such as imidoesters/e.g., as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues may be modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin according to known method steps. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

Tyrosyl residues may be modified by reaction with aromatic diazonium compounds or tetranitromethane. N-acetylimidizol and tetranitromethane may be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides (R'-N-C-N-R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore aspartyl and glutamyl residues may be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues may be frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues may be deamidated under mildly acidic conditions. Either form of these residues falls within the scope of the present invention.

Derivatization with bifunctional agents is useful for cross-linking the peptide to certain chemical moieties. Commonly used cross-linking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 (which are herein incorporated entirely by reference), may be employed for protein immobilization.

Other modifications may include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, *Proteins: Structure and Molecule Properties*, W. H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, methylation of main chain amide residues (or substitution with N-methyl amino acids) and, in some instances, amidation of the C-terminal carboxyl groups, according to known method steps.

Such derivatized molecules may improve the solubility, absorption, permeability across the blood brain barrier biological half life, and the like. Such modifications may alternatively eliminate or attenuate any possible undesirable side effect of the protein and the like. Molecules capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, Pa. (1980).

Such chemical derivatives also may provide attachment to solid supports, including but not limited to, agarose, cellulose, hollow fibers, or other polymeric carbohydrates such as agarose, cellulose, such as for purification, generation of antibodies or cloning; or to provide altered physical properties, such as resistance to enzymatic degradation or increased binding affinity or modulation of IL-13, which is desired for therapeutic compositions comprising IL-13 receptor binding molecules, antibodies thereto or fragments thereof. Such peptide derivatives are well-known in the art, as well as method steps for making such derivatives using carbodiimides active esters of N-hydroxy succinimmide, or mixed anhydrides, as non-limiting examples.

Variation upon the sequences of IL-13 receptor binding molecules of the present invention may also include: the addition of one or more (e.g., two, three, four, or five) lysine, arginine or other basic residues or one, or more (e.g., two, three, four, or five) glutamate or aspartate or other acidic residues at one end of the peptide, where "acidic" and "basic" are as defined herein. Negative charges can also be introduced by the addition of carboxyl, phosphate, borate, sulfonate or sulfate groups. Such modifications may increase the alpha-helical content of the peptide by the "helix dipole effect". They also can provide enhanced aqueous solubility of the peptide, and allow the correct insertion of peptides into a membrane structure.

In another approach, a variety of uncoded or modified amino acids such as D-amino acids and N-methyl amino acids have been used to modify mammalian peptides. Alternatively, a presumed bioactive conformation has been stabilized by a covalent modification, such as cyclization or by incorporation of gamma-lactam or other types of bridges. See, e.g., Veber and Hirschmann, et al., *Proc. Natl. Acad. Sci.* USA, 1978 75 2636 and Thorsett, et al., *Biochem Biophys. Res. Comm.*, 1983 111 166. The primary purpose of such manipulations has not been to avoid metabolism or to enhance oral bioavailability but rather to constrain a bioactive conformation to enhance potency or to induce greater specificity for a receptor subtype.

VII. The Effector Molecule.

As described above, the effector molecule component of the chimeric molecules of this invention may be any molecule whose activity it is desired to deliver to cells that overexpress IL-13 receptors. Particularly preferred effector molecules include cytotoxins such as *Pseudomonas* exotoxin or *Diphtheria* toxin, radionuclides, ligands

such as growth factors, antibodies, detectable labels such as fluorescent or radioactive labels, and therapeutic compositions such as liposomes and various drugs.

A) Cytotoxins.

Particularly preferred cytotoxins include *Pseudomonas* exotoxins, *Diphtheria* toxins, ricin, saponin, gelonin, ribosome inactivating protein, and abrin. *Pseudomonas* exotoxin and *Diphtheria* toxin, modified to remove their capacity for non-specific binding, are the most preferred.

i) *Pseudomonas* exotoxin (PE).

Pseudomonas exotoxin A (PE) is an extremely active monomeric protein (molecular weight 66 kD), secreted by *Pseudomonas aeruginosa*, which inhibits protein synthesis in eukaryotic cells through the inactivation of elongation factor 2 (EF-2) by catalyzing its ADP-ribosylation (catalyzing the transfer of the ADP ribosyl moiety of oxidized NAD onto EF-2).

The toxin contains three structural domains that act in concert to cause cytotoxicity. Domain Ia (amino acids 1-252) mediates cell binding. Domain II (amino acids 253-364) is responsible for translocation into the cytosol and domain III (amino acids 400-613) mediates ADP ribosylation of elongation factor 2, which inactivates the protein and causes cell death. The function of domain Ib (amino acids 365-399) remains undefined, although a large part of it, amino acids 365-380, can be deleted without loss of cytotoxicity. *See* Siegall *et al.*, *J. Biol. Chem.* 264: 14256-14261 (1989).

Where the targeting molecule (*e.g.* an IL-13R binding molecule) is fused to PE, a preferred PE molecule is one in which domain Ia (amino acids 1 through 252) is deleted and amino acids 365 to 380 have been deleted from domain Ib. However all of domain Ib and a portion of domain II (amino acids 350 to 394) can be deleted, particularly if the deleted sequences are replaced with a linking peptide such as GGGGS.

In addition, the PE molecules can be further modified using site-directed mutagenesis or other techniques known in the art, to alter the molecule for a particular desired application. Means to alter the PE molecule in a manner that does not substantially affect the functional advantages provided by the PE molecules described here can also be used and such resulting molecules are intended to be covered herein.

For maximum cytotoxic properties of a preferred PE molecule, several modifications to the molecule are recommended. An appropriate carboxyl terminal

sequence to the recombinant molecule is preferred to translocate the molecule into the cytosol of target cells. Amino acid sequences which have been found to be effective include, REDLK (as in native PE), REDL, RDEL, or KDEL, repeats of those, or other sequences that function to maintain or recycle proteins into the endoplasmic reticulum, referred to here as "endoplasmic retention sequences". *See, for example*, Chaudhary *et al, Proc. Natl. Acad. Sci. USA* 87:308-312 and Seetharam *et al, J. Biol. Chem.* 266: 17376-17381 (1991).

Deletions of amino acids 365-380 of domain Ib can be made without loss of activity. Further, a substitution of methionine at amino acid position 280 in place of glycine to allow the synthesis of the protein to begin and of serine at amino acid position 287 in place of cysteine to prevent formation of improper disulfide bonds is beneficial.

In a preferred embodiment, the targeting molecule is inserted in replacement for domain Ia. A similar insertion has been accomplished in what is known as the TGFα-PE40 molecule (also referred to as TP40) described in Heimbrook *et al., Proc. Natl. Acad. Sci., USA*, 87: 4697-4701 (1990) and in U.S. Patent 5,458,878.

Preferred forms of PE contain amino acids 253-364 and 381-608, and are followed by the native sequences REDLK or the mutant sequences KDEL or RDEL. Lysines at positions 590 and 606 may or may not be mutated to glutamine.

In a particularly preferred embodiment, the IL-13 receptor targeted cytotoxins of this invention comprise the PE molecule designated PE38. This PE molecule is a truncated form of PE composed of amino acids 253-364 and 381-608. Moreover, PE38 can be further modified to create a variant known as PE38QQR by replacing the lysine residues at positions 509 and 606 by glutamine and replacing the residue at 613 by arginine (Debinski *et al. Bioconj. Chem.*, 5: 40 (1994)).

In another particularly preferred embodiment, the IL-13 receptor targeted cytotoxins of this invention comprise the PE molecule designated PE4E. PE4E is a "full length" PE with a mutated and inactive native binding domain where amino acids 57, 246, 247, and 249 are all replaced by glutamates (*see, e.g.,* Chaudhary *et al., J. Biol. Chem.,* 265: 16306 (1995)).

The targeting molecule (*e.g.* the IL-13R binding molecule) may also be inserted at a point within domain III of the PE molecule. Most preferably the targeting molecule is fused between about amino acid positions 607 and 609 of the PE molecule. This means that the targeting molecule is inserted after about amino acid 607 of the molecule and an appropriate carboxyl end of PE is recreated by placing amino acids about

604-613 of PE after the targeting molecule. Thus, the targeting molecule is inserted within the recombinant PE molecule after about amino acid 607 and is followed by amino acids 604-613 of domain III. The targeting molecule may also be inserted into domain Ib to replace sequences not necessary for toxicity. Debinski, *et al. Mol. Cell. Biol.*, 11: 1751-1753 (1991).

In a preferred embodiment, the PE molecules are fused to the targeting molecule by recombinant means. The genes encoding protein chains may be cloned in cDNA or in genomic form by any cloning procedure known to those skilled in the art (*see, e.g.*, Sambrook *et al., Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, (1989)). Methods of cloning genes encoding PE fused to various ligands are well known to those of skill in the art (*see, e.g.,* Siegall *et al., FASEB J.*, 3: 2647-2652 (1989); and Chaudhary *et al. Proc. Natl. Acad. Sci. USA*, 84: 4538-4542 (1987)).

Those skilled in the art will realize that additional modifications, deletions, insertions and the like may be made to the chimeric molecules of the present invention or to the nucleic acid sequences encoding IL-13 receptor-directed chimeric molecules. Especially, deletions or changes may be made in PE or in a linker connecting an antibody gene to PE, in order to increase cytotoxicity of the fusion protein toward target cells or to decrease nonspecific cytotoxicity toward cells without antigen for the antibody. All such constructions may be made by methods of genetic engineering well known to those skilled in the art (see, generally, Sambrook *et al., supra*) and may produce proteins that have differing properties of affinity, specificity, stability and toxicity that make them particularly suitable for various clinical or biological applications.

ii) *Diphtheria* toxin (DT).

Like PE, *Diphtheria* toxin (DT) kills cells by ADP-ribosylating elongation factor 2 thereby inhibiting protein synthesis. *Diphtheria* toxin, however, is divided into two chains, A and B, linked by a disulfide bridge. In contrast to PE, chain B of DT, which is on the carboxyl end, is responsible for receptor binding and chain A, which is present on the amino end, contains the enzymatic activity (Uchida *et al., Science*, 175: 901-903 (1972); Uchida *et al. J. Biol. Chem.*, 248: 3838-3844 (1973)).

In a preferred embodiment, the targeting molecule-*Diphtheria* toxin fusion proteins of this invention have the native receptor-binding domain removed by truncation of the *Diphtheria* toxin B chain. Particularly preferred is DT388, a DT in which the

carboxyl terminal sequence beginning at residue 389 is removed. Chaudhary, *et al.*, *Bioch. Biophys. Res. Comm.*, 180: 545-551 (1991).

Like the PE chimeric cytotoxins, the DT molecules may be chemically conjugated to the IL-13 receptor targeting molecule, but, in a preferred embodiment, the targeting molecule will be fused to the *Diphtheria* toxin by recombinant means. The genes encoding protein chains may be cloned in cDNA or in genomic form by any cloning procedure known to those skilled in the art. Methods of cloning genes encoding DT fused to various ligands are also well known to those of skill in the art (*see, e.g.*, Williams *et al. J. Biol. Chem.* 265: 11885-11889 (1990)).

The term "*Diphtheria* toxin" (DT) as used herein refers to full length native DT or to a DT that has been modified. Modifications typically include removal of the targeting domain in the B chain and, more specifically, involve truncations of the carboxyl region of the B chain.

B) Detectable labels.

Detectable labels suitable for use as the effector molecule component of the chimeric molecules of this invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.* Dynabeads^{TM}), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, texas red, rhodamine, green fluorescent protein, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.*) beads.

Means of detecting such labels are well known to those of skill in the art. Thus, for example, radiolabels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector to detect emitted illumination. Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric labels are detected by simply visualizing the colored label.

C) Ligands.

As explained above, the effector molecule may also be a ligand or an antibody. Particularly preferred ligand and antibodies are those that bind to surface markers on immune cells. Chimeric molecules utilizing such antibodies as effector

molecules act as bifunctional linkers establishing an association between the immune cells bearing binding partner for the ligand or antibody and the tumor cells overexpressing the IL-13 receptor. Suitable antibodies and growth factors are known to those of skill in the art and include, but are not limited to, IL-2, IL-4, IL-6, IL-7, tumor necrosis factor (TNF), anti-Tac, TGFα, and the like.

D) Other therapeutic moieties.

Other suitable effector molecules include pharmacological agents or encapsulation systems containing various pharmacological agents. Thus, the targeting molecule of the chimeric molecule may be attached directly to a drug that is to be delivered directly to the tumor. Such drugs are well known to those of skill in the art and include, but are not limited to, doxirubicin, vinblastine, genistein, an antisense molecule, and the like.

Alternatively, the effector molecule may be an encapsulation system, such as a liposome or micelle that contains a therapeutic composition such as a drug, a nucleic acid (*e.g.* an antisense nucleic acid), or another therapeutic moiety that is preferably shielded from direct exposure to the circulatory system. Means of preparing liposomes attached to proteins are well known to those of skill in the art. See, for example, U.S. Patent No. 4,957,735, Connor *et al.*, *Pharm. Ther.*, 28: 341-365 (1985)

VIII. Attachment of the Targeting Molecule to the Effector Molecule.

One of skill will appreciate that the targeting molecule and effector molecules may be joined together in any order. Thus, where the targeting molecule is a polypeptide, the effector molecule may be joined to either the amino or carboxy termini of the targeting molecule. The targeting molecule may also be joined to an internal region of the effector molecule, or conversely, the effector molecule may be joined to an internal location of the targeting molecule, as long as the attachment does not interfere with the respective activities of the molecules.

The targeting molecule and the effector molecule may be attached by any of a number of means well known to those of skill in the art. Typically the effector molecule is conjugated, either directly or through a linker (spacer), to the targeting molecule. However, where both the effector molecule and the targeting molecule are polypeptides it is preferable to recombinantly express the chimeric molecule as a single-chain fusion protein.

A) Conjugation of the effector molecule to the targeting molecule.

In one embodiment, the targeting molecule (*e.g.*, IL-13R binding molecule) is chemically conjugated to the effector molecule (*e.g.*, a cytotoxin, a label, a ligand, or a drug or liposome). Means of chemically conjugating molecules are well known to those of skill.

The procedure for attaching an agent to an antibody or other polypeptide targeting molecule will vary according to the chemical structure of the agent. Polypeptides typically contain variety of functional groups; *e.g.*, carboxylic acid (COOH) or free amine (-NH_2) groups, which are available for reaction with a suitable functional group on an effector molecule to bind the effector thereto.

Alternatively, the targeting molecule and/or effector molecule may be derivatized to expose or attach additional reactive functional groups. The derivatization may involve attachment of any of a number of linker molecules such as those available from Pierce Chemical Company, Rockford Illinois.

A "linker", as used herein, is a molecule that is used to join the targeting molecule to the effector molecule. The linker is capable of forming covalent bonds to both the targeting molecule and to the effector molecule. Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide linkers. Where the targeting molecule and the effector molecule are polypeptides, the linkers may be joined to the constituent amino acids through their side groups (*e.g.*, through a disulfide linkage to cysteine). In a preferred embodiment, however, the linkers will be joined to the alpha carbon amino and carboxyl groups of the terminal amino acids.

A bifunctional linker having one functional group reactive with a group on a particular agent, and another group reactive with an antibody, may be used to form a desired immunoconjugate. Alternatively, derivatization may involve chemical treatment of the targeting molecule, *e.g.*, glycol cleavage of the sugar moiety of a the glycoprotein antibody with periodate to generate free aldehyde groups. The free aldehyde groups on the antibody may be reacted with free amine or hydrazine groups on an agent to bind the agent thereto. (See U.S. Patent No. 4,671,958). Procedures for generation of free sulfhydryl groups on polypeptide, such as antibodies or antibody fragments, are also known (See U.S. Pat. No. 4,659,839).

Many procedure and linker molecules for attachment of various compounds including radionuclide metal chelates, toxins and drugs to proteins are

known. See, for example, European Patent Application No. 188,256; U.S. Patent Nos. 4,671,958, 4,659,839, 4,414,148, 4,699,784; 4,680,338; 4,569,789; and 4,589,071; and Borlinghaus *et al. Cancer Res.* 47: 4071-4075 (1987).

In some circumstances, it is desirable to free the effector molecule from the targeting molecule when the chimeric molecule has reached its target site. Therefore, chimeric conjugates comprising linkages which are cleavable in the vicinity of the target site may be used when the effector is to be released at the target site. Cleaving of the linkage to release the agent may be prompted by enzymatic activity or conditions to which the chimeric molecule is subjected either inside the target cell or in the vicinity of the target site. When the target site is a tumor, a linker which is cleavable under conditions present at the tumor site (*e.g.* when exposed to tumor-associated enzymes or acidic pH) may be used.

A number of different cleavable linkers are known to those of skill in the art. See, U.S. Pat. Nos. 4,618,492; 4,542,225, and 4,625,014. The mechanisms for release of an agent from these linker groups include, for example, irradiation of a photolabile bond and acid-catalyzed hydrolysis. U.S. Pat. No. 4,671,958, for example, includes a description of immunoconjugates comprising linkers which are cleaved at the target site *in vivo* by the proteolytic enzymes of the patient's complement system. In view of the large number of methods that have been reported for attaching a variety of radiodiagnostic compounds, radiotherapeutic compounds, drugs, toxins, and other agents to antibodies, one skilled in the art will be able to determine a suitable method for attaching a given agent to a polypeptide.

B) Production of fusion proteins.

Where the targeting molecule and/or the effector molecule is relatively short (*i.e.*, less than about 50 amino acids) they may be synthesized using standard chemical peptide synthesis techniques. Where both molecules are relatively short the chimeric molecule may be synthesized as a single contiguous polypeptide. Alternatively the targeting molecule and the effector molecule may be synthesized separately and then fused by condensation of the amino terminus of one molecule with the carboxyl terminus of the other molecule thereby forming a peptide bond. Alternatively, the targeting and effector molecules may each be condensed with one end of a peptide spacer molecule thereby forming a contiguous fusion protein.

Solid phase synthesis in which the C-terminal amino acid of the sequence is attached to an insoluble support followed by sequential addition of the remaining amino acids in the sequence is the preferred method for the chemical synthesis of the polypeptides of this invention. Techniques for solid phase synthesis are described by Barany and Merrifield, *Solid-Phase Peptide Synthesis*; pp. 3-284 in *The Peptides: Analysis, Synthesis, Biology. Vol. 2: Special Methods in Peptide Synthesis, Part A.*, Merrifield, *et al. J. Am. Chem. Soc.*, 85: 2149-2156 (1963), and Stewart *et al., Solid Phase Peptide Synthesis, 2nd ed.* Pierce Chem. Co., Rockford, Ill. (1984).

In a preferred embodiment, the chimeric fusion proteins of the present invention are synthesized using recombinant DNA methodology. Generally this involves creating a DNA sequence that encodes the fusion protein, placing the DNA in an expression cassette under the control of a particular promoter, expressing the protein in a host, isolating the expressed protein and, if required, renaturing the protein.

DNA encoding the fusion proteins (*e.g.* IL-13-PE38QQR) of this invention may be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences or direct chemical synthesis by methods such as the phosphotriester method of Narang *et al. Meth. Enzymol.* 68: 90-99 (1979); the phosphodiester method of Brown *et al., Meth. Enzymol.* 68: 109-151 (1979); the diethylphosphoramidite method of Beaucage *et al., Tetra. Lett.*, 22: 1859-1862 (1981); and the solid support method of U.S. Patent No. 4,458,066.

Chemical synthesis produces a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill would recognize that while chemical synthesis of DNA is limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

Alternatively, subsequences may be cloned and the appropriate subsequences cleaved using appropriate restriction enzymes. The fragments may then be ligated to produce the desired DNA sequence.

In a preferred embodiment, DNA encoding fusion proteins of the present invention may be cloned using DNA amplification methods such as polymerase chain reaction (PCR). Thus, in a preferred embodiment, the gene for IL-13 is PCR amplified, using a sense primer containing the restriction site for NdeI and an antisense primer containing the restriction site for HindIII. In a particularly preferred embodiment, the

primers are selected to amplify the nucleic acid starting at position 19, as described by McKenzie *et al.* (1987), *supra.* This produces a nucleic acid encoding the mature IL-13 sequence and having terminal restriction sites. A PE38QQR fragment may be cut out of the plasmid pWDMH4-38QQR or plasmid pSGC242FdN1 described by Debinski *et al. Int. J. Cancer*, 58: 744-748 (1994), and by Debinski *et al. Clin. Res.*, 42: 251A (abstract (1994) respectively. Ligation of the IL-13 and PE38QQR sequences and insertion into a vector produces a vector encoding IL-13 joined to the amino terminus of PE38QQR (position 253 of PE). The two molecules are joined by a three amino acid junction consisting of glutamic acid, alanine, and phenylalanine introduced by the restriction site.

While the two molecules are preferably essentially directly joined together, one of skill will appreciate that the molecules may be separated by a peptide spacer consisting of one or more amino acids. Generally the spacer will have no specific biological activity other than to join the proteins or to preserve some minimum distance or other spatial relationship between them. However, the constituent amino acids of the spacer may be selected to influence some property of the molecule such as the folding, net charge, or hydrophobicity.

The nucleic acid sequences encoding the fusion proteins may be expressed in a variety of host cells, including *E. coli,* other bacterial hosts, yeast, and various higher eukaryotic cells such as the COS, CHO and HeLa cells lines and myeloma cell lines. The recombinant protein gene will be operably linked to appropriate expression control sequences for each host. For *E. coli* this includes a promoter such as the T7, trp, or lambda promoters, a ribosome binding site and preferably a transcription termination signal. For eukaryotic cells, the control sequences will include a promoter and preferably an enhancer derived from immunoglobulin genes, SV40, cytomegalovirus, etc., and a polyadenylation sequence, and may include splice donor and acceptor sequences.

The plasmids of the invention can be transferred into the chosen host cell by well-known methods such as calcium chloride transformation for *E. coli* and calcium phosphate treatment or electroporation for mammalian cells. Cells transformed by the plasmids can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as the amp, gpt, neo and hyg genes.

Once expressed, the recombinant fusion proteins can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (*see*, generally, R. Scopes, *Protein Purification*, Springer-Verlag, N.Y. (1982), Deutscher, *Methods in*

Enzymology Vol. 182: Guide to Protein Purification., Academic Press, Inc. N.Y. (1990)). Substantially pure compositions of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity are most preferred for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically.

One of skill in the art would recognize that after chemical synthesis, biological expression, or purification, the IL-13 receptor targeted fusion protein may possess a conformation substantially different than the native conformations of the constituent polypeptides. In this case, it may be necessary to denature and reduce the polypeptide and then to cause the polypeptide to re-fold into the preferred conformation. Methods of reducing and denaturing proteins and inducing re-folding are well known to those of skill in the art (See, Debinski *et al. J. Biol. Chem.*, 268: 14065-14070 (1993); Kreitman and Pastan, *Bioconjug. Chem.*, 4: 581-585 (1993); and Buchner, *et al., Anal. Biochem.*, 205: 263-270 (1992)). Debinski *et al.*, for example, describe the denaturation and reduction of inclusion body proteins in guanidine-DTE. The protein is then refolded in a redox buffer containing oxidized glutathione and L-arginine.

One of skill would recognize that modifications can be made to the IL-13 receptor targeted fusion proteins without diminishing their biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of the targeting molecule into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids placed on either terminus to create conveniently located restriction sites or termination codons.

IX. Identification of Target Cells.

Tumor cells overexpress IL-13 receptors. In particular, carcinoma tumor cells (*e.g.* renal carcinoma cells) overexpress IL-13 receptors at levels ranging from about 2100 sites/cell to greater than 150,000 sites per cell. Similarly, gliomas and Kaposi's sarcoma also overexpress IL-13 receptors (IL-13R). Moreover, at least some cells of virtually every cancer type tested to date appear to overexpress IL-13 receptors. For example, overexpression of IL-13R has been found in some pancreatic cancers and about one-quarter of the head and neck cancers studied. Thus it appears that IL-13 receptor overexpression is a general characteristic of many solid tumor neoplastic cells.

Thus, the methods of this invention can be used to target an effector molecule to IL-13R-overexpressing neoplastic cells. Neoplasias are well known to those of skill in the art and include, but are not limited to, cancers of the skin (*e.g.,* basal or squamous cell carcinoma, melanoma, Kaposi's sarcoma, *etc.*), cancers of the reproductive system (*e.g.,* testicular, ovarian, cervical), cancers of the gastrointestinal tract (*e.g.*, stomach, small intestine, large intestine, colorectal, *etc.*), cancers of the mouth and throat (*e.g.* esophageal, larynx, oropharynx, nasopharynx, oral, *etc.*), cancers of the head and neck, bone cancers, breast cancers, liver cancers, prostate cancers (*e.g.*, prostate carcinoma), thyroid cancers, heart cancers, retinal cancers (*e.g.*, melanoma), kidney cancers, lung cancers (*e.g.*, mesothelioma), pancreatic cancers, brain cancers (*e.g.* gliomas, medulloblastomas, pituitary adenomas, *etc.*) and cancers of the lymph system (*e.g.* lymphoma).

In a particularly preferred embodiment, the methods of this invention are used to target effector molecules to kidney cancers, colorectal cancers (especially colorectal carcinomas), to skin cancers (especially Kaposi's sarcoma), and to brain cancers (especially gliomas, and medulloblastomas).

One of skill in the art will appreciate that identification and confirmation of IL-13 overexpression by cells requires only routine screening using well-known methods. Typically this involves providing a labeled molecule that specifically binds to the IL-13 receptor. The cells in question are then contacted with this molecule and washed. Quantification of the amount of label remaining associated with the test cell provides a measure of the amount of IL-13 receptor (IL-13R) present on the surface of that cell.

In a preferred embodiment, the IL-13 receptor present may be quantified by measuring the binding of ^{125}I-labeled IL-13 (^{125}I-IL-13) to the cell in question. Details of such a binding assay are provided in the Examples.

X. Pharmaceutical Compositions

The antagonists, agonists, and chimeric molecules of the invention are useful for parenteral, topical, oral, or local administration, such as by aerosol or transdermally, for prophylactic and/or therapeutic treatment. Compositions of these molecules can be administered in a pharmaceutically acceptable carrier in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include powder, tablets, pills, capsules and

lozenges. It is recognized that the antagonists, agonists, and fusion proteins of this invention are peptides or proteins and, when administered orally, must be protected from digestion. This is typically accomplished either by complexing the peptide or protein with a composition to render it resistant to acidic and enzymatic hydrolysis or by packaging the molecule in an appropriately resistant carrier such as a liposome. Means of protecting peptides or proteins from digestion are well known in the art.

The pharmaceutical compositions of this invention are particularly useful for parenteral administration, such as intravenous administration or administration into a body cavity or lumen of an organ. The compositions for administration will commonly comprise a solution of the antagonist, agonist, or chimeric molecule dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, *e.g.*, buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of antagonists, agonists, or chimeric molecule in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the patient's needs.

Thus, a typical pharmaceutical composition for intravenous administration would be about 0.1 to 10 mg per patient per day. Dosages from 0.1 up to about 100 mg per patient per day may be used, particularly when the drug is administered to a secluded site and not into the blood stream, such as into a body cavity or into a lumen of an organ. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in such publications as *Remington's Pharmaceutical Science*, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980).

The compositions containing the peptides or proteins of the invention or a cocktail thereof (*i.e.*, with other proteins) can be administered for therapeutic treatments. In therapeutic applications, compositions are administered to a patient suffering from a disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically

effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the patient's health.

Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the patient. In any event, the composition should provide a sufficient quantity of the proteins of this invention to effectively treat the patient.

Among various uses of the cytotoxic chimeric molecules, such as fusion proteins, of the present invention are included inhibiting a variety of disease conditions caused by specific human cells that may be eliminated by the toxic action of the protein. One preferred application is the inhibition of the growth of cancer cells, such as by the use of an IL-13 receptor binding molecule of the invention attached to a cytotoxin which kills or inhibits growth of cells which are contacted by or, especially, bound by the chimeric molecule.

Where the chimeric molecule comprises an IL-13 receptor targeting molecule attached to a ligand, the ligand portion of the molecule is chosen according to the intended use. Proteins on the membranes of T cells that may serve as targets for the ligand includes CD2 (T11), CD3, CD4 and CD8. Proteins found predominantly on B cells that might serve as targets include CD10 (CALLA antigen), CD19 and CD20. CD45 is a possible target that occurs broadly on lymphoid cells. These and other possible target lymphocyte target molecules for the chimeric molecules bearing a ligand effector are described in *Leukocyte Typing III*, A.J. McMichael, ed., Oxford University Press (1987). Those skilled in the art will realize ligand effectors may be chosen that bind to receptors expressed on still other types of cells as described above, for example, membrane glycoproteins or ligand or hormone receptors such as epidermal growth factor receptor and the like.

It will be appreciated by one of skill in the art that there are some regions that are not heavily vascularized or that are protected by cells joined by tight junctions and/or active transport mechanisms which reduce or prevent the entry of macromolecules present in the blood stream. Thus, for example, systemic administration of therapeutics to treat gliomas, or other brain cancers, is constrained by the blood-brain barrier which resists the entry of macromolecules into the subarachnoid space.

One of skill in the art will appreciate that in these instances, the therapeutic compositions of this invention can be administered directly to the tumor site. Thus, for example, brain tumors (*e.g.*, gliomas) can be treated by administering the therapeutic

composition directly to the tumor site (*e.g.*, through a surgically implanted catheter). Where the fluid delivery through the catheter is pressurized, small molecules (*e.g.* the therapeutic molecules of this invention) will typically infiltrate as much as two to three centimeters beyond the tumor margin.

Alternatively, the therapeutic composition can be placed at the target site in a slow release formulation. Such formulations can include, for example, a biocompatible sponge or other inert or resorbable matrix material impregnated with the therapeutic composition, slow dissolving time release capsules or microcapsules, and the like.

Typically the catheter or time release formulation will be placed at the tumor site as part of a surgical procedure. Thus, for example, where major tumor mass is surgically removed, the perfusing catheter or time release formulation can be emplaced at the tumor site as an adjunct therapy. Of course, surgical removal of the tumor mass may be undesired, not required, or impossible, in which case, the delivery of the therapeutic compositions of this invention may comprise the primary therapeutic modality.

XI. Diagnostic Kits.

In another embodiment, this invention provides for kits for inhibiting the growth of tumors or for the detection of cells overexpressing IL-13 receptors. Kits will typically comprise a chimeric molecule of the present invention (*e.g.* IL-13R binding molecule-label, IL-13R binding molecule -cytotoxin, IL-13 R binding molecule -ligand, *etc.*). In addition the kits will typically include instructional materials disclosing means of use of chimeric molecule (*e.g.* as a cytotoxin, for detection of tumor cells, to augment an immune response, *etc.*). The kits may also include additional components to facilitate the particular application for which the kit is designed. Thus, for example, where a kit contains a chimeric molecule in which the effector molecule is a detectable label, the kit may additionally contain means of detecting the label (*e.g.* enzyme substrates for enzymatic labels, filter sets to detect fluorescent labels, appropriate secondary labels such as a sheep anti- mouse-HRP, or the like). The kits may additionally include buffers and other reagents routinely used for the practice of a particular method. Such kits and appropriate contents are well known to those of skill in the art.

polyacrylamide gel at 150 V for 2.5 hr. Gels were dried for 2 hr and autoradiographed overnight at room temperature.

CD14 regulation by IL-13

Flow cytometric analysis of human primary monocytes was performed as described elsewhere (Cosentino, G. et al., *J. Immunol.* **155**:3145-3151 (1995); Oshima, Y. et al., *J. Biol. Chem.* **275**:14375-14380 (2000)). Primary monocytes were cultured at $1X10^7$ cells /ml in polypropylene tubes for 48 h with 1ng/ml wtIL-13 with or without 1μg/ml *IL-13E13KR112D.* Cells were washed and incubated at 4 °C for 60 min in FACS staining buffer (HBSS containing 0.5% FBS, 0.1 % sodium azide) containing FITC-conjugated anti-human CD14 (Becton Dickinson, San Jose, CA) antibodies as per the manufacture's recommendations. For controls, cells were either incubated in FACS staining buffer alone or with isotype control antibody, mouse IgG2a and then antimouse Ig FITC-conjugated was used as secondary antibody for staining. The cells were subsequently washed, and fluorescence data were collected on a FACScan/C32 equipment (Becton Dickinson, San Jose, CA). The results were analyzed with a CELLQuest (Becton Dickinson, San Jose, CA) program, and fluorescence intensity was expressed as mean channel number (MCN) on 256 channel/10^4 log scale.

Example 17: Comparison of Activity of IL-13R112D and IL-13E13KR112D

Recombinant protein isolation and purification

Recombinant wtIL-13, *IL-13R112D* and IL-13 double mutein, *IL-13E13KR112D* were expressed in *Escherichia coli* and purified from inclusion bodies. After purification, each recombinant protein was analyzed using SDS-PAGE and stained with Coomassie blue. Visual inspection of bands suggested that purity of all preparation was more than 95 %.

IL-13E13KR112D competes for the binding of radiolabeled IL-13

Binding studies were performed on the PM-RCC renal cell carcinoma cell line and the U251 glioblastoma cell line. Both cell lines express type I IL-13 receptor (Obiri, N. I. et al., *J. Immunol.* **158**:756-764 (1997); Murata, T. et al., *Int. J. Mol. Med.* 1:551-557 (1998)). Both cell lines showed similar results. As expected, wtIL-13 displaced specific binding of radiolabeled IL-13. Double mutein IL-13 also inhibited

EXAMPLES

The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1 : Materials

Restriction endonucleases and DNA ligase were obtained from New England Biolabs (Beverly, MA), BRL (Gaithersburg, MD), PANVERA (Madison, WI) and Boehringer Mannheim (Indianapolis, IN). Fast Protein Liquid Chromatographic (FPLC) columns and media were purchased from Pharmacia (Piscataway, NJ). Oligonucleotide primers were synthesized at Bioserve Biotechnologies (Laurel, MD). Advantage-HF Polymerase chain reaction (PCR) kit was from Clontech (Palo Alto, CA).

All plasmids carrying the genomes encoding the IL13 proteins were under a T7 bacteriophage late promoter, a T7 transcription terminator at the end of the open reading frame and a f1 origin of replication and gene for ampicillin resistance. Plasmids were amplified in *E. coli* (DH5alpha high efficiency transformation) (BRL) and DNA was extracted using Qiagen kits (Chatsworth, CA). TF-1 human erythroleukemia cell line was obtained from the ATCC (Manassas, VA) and were grown in human GM-CSF. B9 mouse plasmacytoma cell line was a kind gift of Dr. Lucine Aaden and were grown in human hIL-6. PM-RCC renal cell carcinoma cell line was established in our laboratory. (Puri, R.K. *et al.*, *Cellular Immunology* 171:80-86 (1996); Puri, R.K. *et al.*, *Blood* 87:4333-4339 (1996); Obiri, N.I. *et al.*, *J Clin Investig* 91:88-93 (1993). Primary monocytes and THP-1 cells were kindly provided by Dr. Subhash Dhawan (CBER FDA) and Dr. Ray Donnelley (CBER FDA), respectively. Buffycoat from peripheral blood were obtained from healthy volunteers who donated blood at NIH Blood Bank. Mononuclear cells were isolated by centrifugation over Ficoll-Paque Plus (Pharmacia Biotech).

Example 2: Protein Homology Search and Prediction of Secondary Structure

Homology search and secondary structure analysis of IL-13: A computer program, GCG (Genetics Computer Group, Inc., Madison, WI) was used for homology search, data base search, and prediction of secondary structure of IL-13 on Silicon Graphics Workstation in Human Genome Center, the Institutes of Medical Science, the University of Tokyo (Tokyo, Japan) and the Center for Information Technology, National

Institutes of Health (Bethesda, MD). The protein sequence of mouse (il13_mouse.swissprot), rat (l26913.gb_ro), human (Caf043334_1.gp_main) and bos taurus (bta132441_1.gp_main) were obtained from Swiss Prot and GenBank. Since l26913.gb_ro is not the protein sequence but a cDNA sequence, it was used for homology searching after first being translated into the corresponding protein sequence. The IL13 sequence is shown after deletion of predicted signal sequence of *Homo sapiens* or equivalent [Fig. 1]. Hydrophobicity and secondary structure of IL13 was predicted by Kyte-Doolittle method and Chou-Fasman method respectively. (Chou, P. *et al.*, Biochemistry 13:222-245 (1974)).

Example 3: Construction of plasmids encoding IL-13R112D

The mutagenesis of IL13 gene was performed using a cDNA as a template using sense primer 5'- taa ttt gcc cat atg tcc cca ggc cct gtg cct -3', anti-sense primer 5'- taa ttt gcc cga att cag ttg aag tct ccc tcg cg -3' to mutate Arg112 to Asp112 and to incorporate NdeI and EcoRI restriction enzyme sites at the 5'- and 3'- termini, respectively. After subcloning the PCR products into pCR2.1 (Invitrogen), the plasmid was digested with NdeI and EcoRI. The fragment was inserted into a prokaryotic expression vector, pG420, digested with same restriction enzymes. We confirmed the existence of mutation and restriction sites by sequencing of the plasmid.

Example 4: Expression and purification of recombinant proteins

Expression and purification of IL-13R112D and wtIL-13 was carried out by essentially the same techniques as previously reported for IL4. (Kreitman, R.J. *et al.*, Cytokine 7:311-318 (1995)). However, instead of BL21(lambdaDE3) *E. coli*, we used BL21(lambdaDE3)pLys *E. coli* genome that contains T7 RNA polymerase under the lac promoter and lac operator. The protein expression was induced by adding 1mM IPTG. WtIL13 and IL-13R112D were produced in inclusion bodies, which contained a major, rather pure protein of 13 kDa. After washing, inclusion bodies were solubilized, refolded and purified by FPLC ion-exchange chromatography. The resulting protein was highly purified (> 95% pure) and showed one thick band at 13 kDa in Coomassie Blue stained SDS- polyacrylamide-gels after electrophoresis. To confirm the identity, the protein was shown to react with anti-human IL-13 antibodies on Western blots. We also observed a

minor 26 kDa protein in SDS-PAGE gels that we regarded as dimerized IL13R112D or wtIL13.

Example 5: Cell Proliferation Assays

Proliferation assays were performed as described previously. (Leland, P. *et al.*, Oncology Research 7:227-235 (1995)). TF-1 and B9 cells were washed 2-3 times to remove GM-CSF and IL-6 and then $1X10^3$ to $5X10^3$ cells were cultured in 96-well plates in RPMI complete medium containing 10 % fetal bovine serum. Varying concentration of wtIL-13 and IL-13R112D were added to the wells, and the cells were cultured for 1-2 days. Tritiated thymidine (0.5 μCi) was added to each well 6-9 hours before the plates were harvested in a Skatron cell harvester (Skatron, Inc., Sterling, VA). Filter mats were counted in a beta plate counter (Wallac).

Example 6: Protein Synthesis Inhibition Assay

Protein synthesis inhibition assay was performed as previously described. (Puri, R.K., Cancer Research 51:6209-6209 (1991); Puri, R.K. *et al.*, Cancer Research 51 :3011-3017 (1991)). In brief, $1X10^3$ PM-RCC cells were cultured in leucine-free medium (Biofluids, Rockville, MD) for 4 h to allow adherence to flat-bottomed microtiter plates. These cells then received various concentrations of IL-13-PE38, incubated for 20-24 h at 37 °C and then 1 μCi of ^{3}H-leucine (NEN, Boston, MA) was added to each well and cultured for an additional 4 h. For blocking experiments, 2000 ng/ml of wt-IL-13 or IL-13R112D was added prior to the addition of IL13-PE38. Finally, cells were washed and harvested on fiberglass filtermat and cell associated radioactivity was measured in a Beta Plate Counter (Wallac, Gaithersburg, MD). The concentration of IL-13-PE38 at which 50 % inhibition of protein synthesis (IC50) occurred was calculated.

Example 7: IL-13 Receptor Binding Studies

Recombinant human IL-4 and rhIL-13 were labeled with 125I (Amersham) by using IODO-GEN reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. The specific activity of radiolabeled IL-4 and IL-13 ranged

from 21.0 to 22.0 μCi/μg and 17.6 to 18.0 μCi/μg, respectively. The equilibrium binding studies were carried out as described elsewhere. (Obiri, N.I. *et al.*, *J Clin Investig* 91:88-93 (1993); Husain, S.R. et al., *Molecular Medicine* 3, no. 5:327-338 (1997); Obiri, N.I. et al., *J Biol Chem* 270:8797-8804 (1995); Obiri, N.I. et al., *J Immunol* 158:756-764 (1997)). Briefly, 1X10^6 cells in 100 μl of binding buffer were incubated at 4 °C for 2 hr with ^{125}I-IL-4 (500 pM) or ^{125}I-IL-13 (500 pM) in the absence or presence of increasing concentrations (10 pM - 200 nM) of unlabeled wt IL-13 or IL-13R112D. The duplicate samples of the cells associated with 125I-IL-4 or 125I-IL-13 were separated from free ^{125}I-IL-4 or ^{125}I-IL-13 by centrifugation through cushion of phthalate oils. The cell pellets were counted in a Gamma-counter (Wallac, Gaithersburg, MD).

Example 8: Flow Cytometry

Flow cytometric analysis of monocytes were performed as described elsewhere. (Cosentino, G. et al., J Immunol 155:3145-3151 (1995)). Primary monocytes were cultured at 1X10^7 cells /ml in polypropylene tubes for 72 h with various concentration of wt-IL-13 or IL-13R112D. Cells were washed and incubated at 4°C for 60 min in FACS staining buffer (HBSS containing FBS, 0.1 % sodium azide) containing FITC-conjugated anti-human CD14 (Becton Dickinson, San Jose, CA) antibodies at a concentration as recommended by manufacturer. For controls, cells were either incubated in FACS staining buffer alone or with isotype control antibody IgG2a, and antimouse Ig FITC-conjugate was then used as a secondary antibody for staining. The cells were subsequently washed, and fluorescence data were collected on a FACScan/C32 (Becton Dickinson, San Jose, CA). The data were analyzed with a WinList software program, and fluorescence intensity was expressed as mean cannel number (MCN) on 256 channel/10^4 log scale.

Example 9: Electrophoretic mobility-shift assay (EMSA)

EMSA was performed as described before. (Murata, T. et al., *Intl J Cancer* 70:230-240 (1997);Murata, T. et al., *Blood* 91, no. 10:3884-3891 (1998)). After incubation with various concentrations of wt IL-13 or IL-13R112D for 10 minutes, THP-1 cells, primary monocytes and Tory EBV immortalized B cells (a gift from Dr. Giovanna Tosato, CBER/FDA) were washed with cold PBS and solubilized with cold whole-cell

extraction buffer (1 mM $MgCl_2$, 20 mM HEPES, pH 7.0, 10 mM KCl, 300 mM NaCl, 0.5 mM dithiothreitol, 0.1% NP-40, 1 mM PMSF, 1 mM Na_3 VO_4 and 20% glycerol). DNA-protein interactions were assessed by EMSA using the Bandshift kit from Pharmacia. Briefly, 40 μg of sample proteins were incubated for 20 min. at room temperature with 1 ng of 32 P-labeled double stranded oligonucleotide probe SBE-1 (sense oligo 5'-gAT CgC TCT TCT TCC CAg gAA CTC AAT g-3, anti sense oligo 5'-TCg ACA TTg AgT TCC Tgg gAA gAA gAg C-3') in binding buffer [10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 10% glycerol, 0.05% NP-40, 0.05 mg/ml poly (dI-dC)2]. In some experiment, a 200-fold excess of cold SBE-1 probe was added as a competitor, and 2 μl of loading dye was added to samples which were then electrophoresed in a 5% non-reducing polyacrylamide gel at 150 V for 2 hr. Gels were dried for 2 hr and autoradiographed overnight at room temperature.

Example 10: Proliferation activity of wtIL-13 and IL-13R112D on hematopoietic cell lines

After purification of IL13 and mutated IL13, the goal was to compare their biological activities on various cell types that express different types of IL13R. First, we tested the mitogenic activity of these two forms of IL-13. IL13 has been shown to induce proliferation of TF-1 human erythroleukemia cell line. (Kitamura, T. et al., *J Cellular Physiol* 140:323-334 (1989); Kitamura, T. et al., *Blood* 73:375-380 (1989)). We tested the proliferative activity of IL-13R112D on the TF-1 cell line. The proliferative activity of IL13R112D was more than 10 times better than that wtIL-13. The concentration of wtIL13 that produced half maximal proliferation (ED50) was about 2 ng/ml, compared to less than 0.2 ng/ml for IL13R112D. Similarly, IL-13R112D stimulated mouse plasmacytoma cell line B9 much more strongly than did wtIL-13. IL-13R112D was 5.7-19 fold better than wtIL-13 in proliferation assays. Thus, the proliferation activity of IL-13R112D on hematopoietic cells which express the Type III IL13 receptor complex is about one log better than wtIL-13.

Example 11: IL13R112D suppresses CD14 expression on monocytes more strongly than does wtIL-13:

Since IL-13 has been shown to downregulate CD14 expression on

monocytes (Cosentino, G. et al., *J Immunol,* 155:3145-3151 (1995)), we investigated whether the IL13R112D mutant has stronger activity than does wtIL13. We found that IL-13R112D and wtIL-13 suppressed CD14 expression on monocytes in a dose dependent manner. IL13R112D was superior than wtIL13 in downregulation of CD14. For example, 1 ng/ml IL-13R112D induced downregulation of CD14 which was similar to that induced by 10 ng/ml wtIL13. In addition 10 ng/ml IL13R112D produced similar downregulation as did 50 ng/ml wtIL13.

Example 12: STAT 6 activation in THP-1 cells, monocytes and EBV-immortalized B cells

IL13 has been shown to phosphorylate and activate STAT6 protein for signal transduction in various cell types. (Murata, T. et al., *J Immunol* 156, no. 8:2972-2978 (1996); Obiri, N.I. et al., *J Biol Chem* 272, no. 32:20251-20258 (1997); Murata, T. et al., *Cellular Immunology* 175:33-40 (1997); Murata, T. et al., *International J Cancer* 70, no. 2:230-240 (1997); Murata, T. et al., *International Immunology* 10:1103-1110 (1998); Murata, T. et al., *Blood* 91:3884-3891 (1998)). Therefore, we compared the ability of wtIL-13 and of IL-13R112D to stimulate STAT6 in a monocytic cell line, THP-1, which expresses the Type II receptor complex, and in primary monocytes which express Type III receptor complex. (Murata, T. et al., *Intl J Mol Med* 1:551-557 (1998)). The concentration at which IL-13R112D and wtIL-13 stimulated maximal activation of STAT6 of primary monocytes was 10 ng/ml and 50 ng/ml, respectively. However, 1 ng/ml of IL-13R112D stimulated maximal activation of STAT6 in THP-1 cells compared to 10 ng/ml wtIL-13 that stimulated a slightly less than maximal activation. These studies demonstrate that IL13R112D has approximately 5-10 times better activity than wtIL13 on human monocytes.

Example 13: Inhibition of [^{125}I]IL13 and [^{125}I]IL4 binding by IL13 mutant

We tested the ability of the IL13 mutant to replace [^{125}I]IL-13 and [^{125}I]IL-4 binding in PM-RCC cells which express the Type I IL13 receptor complex. The concentration of IL-13R112D that inhibited [^{125}I]IL13 binding by 50% (ED50) was 150 pM, compared to 650 pM by wtIL13. On the other hand, the ED50 of wtIL-13 and IL-13R112D to replace [^{125}I]IL-4 binding was 800 pM and 100 pM, respectively. Thus, IL-

13R112D interacted more strongly with the IL4R and the IL13R than did wtIL-13.

Example 14: Inhibition of IL13-toxin-mediated cytotoxicity by IL13 mutant:

We have previously demonstrated that an IL13-cytotoxin (IL13-PE38) is specifically and highly cytotoxic to a PM-RCC cell line. (Puri, R.K. et al., *Blood* 87:4333-4339 (1996)). To determine the relative ability of the IL13 mutant and wtIL13 to act as the targeting moiety of a chimeric molecule bearing a cytotoxin, we compared the ability of wtIL-13 and of IL-13R112D to displace cytotoxicity mediated by IL-13-PE38 in PM-RCC cells. IL-13R112D appeared to be better than wtIL13 in blocking cytotoxicity of IL13-toxin. IL13PE38 was highly cytotoxic to these cells with a concentration that inhibited protein synthesis by 50% (IC50) of less than 0.1 ng/ml. In the presence of 2000 ng/ml wtIL13, the IC50 increased to 60 ng/ml while in the presence of IL13R112D, the IC50 reached 105 ng/ml. To carefully determine the extent of superiority of IL13 mutant in blocking cytotoxicity of IL13PE38, we used varying concentration of cytotoxins in the presence of a fixed concentration of IL13PE38. In this assay, the IL13 mutant was approximately 10 times better than wtIL13 in blocking the cytotoxicity.

Example 15: Loading of Antigen Presenting Cells with Antigen

Peripheral blood mononuclear cells (PBMCs) are isolated from a patient and cultured in RPMI-1640 medium. The cells are incubated with GM-CSF and IL-13 in which the arginine at position 112 has been mutated to an aspartic acid. Tumor cells from the patient are lysed and lysate representing approximately 10^6 tumor cells is added to the medium to pulse the cells with tumor antigen. The cells are incubated with the lysate for 4 hours. The cells are then washed with fresh medium and an aliquot is tested to ensure presentation of the antigen by the cells. Testing is conducted using a mixed leukocyte reaction. The aliquot of pulsed antigen presenting cells (APCs) are placed in a well of a standard 96-well plate, to which are added T cells from a second donor. The T cells are incubated with the pulsed APCs for 4 hours and then tested to see if they become lytic to cells expressing the antigen with which the APCs were pulsed. Testing of the T cells is performed by standard ^{51}Chromium release or cytokine release assays.

Example 16: Materials and Methods for Example 17

This Example sets forth materials and methods used in Example 17.

Materials

Restriction endonucleases and DNA ligase were obtained from New England Biolabs (Beverly, MA), BRL (Gaithersburg, MD), PANVERA (Madison, WI) and Boehringer Mannheim (Indianapolis, IN). Fast Protein Liquid Chromatographic (FPLC) columns and media were purchased from Pharmacia (Piscataway, NJ). Sequence specific oligonucleotide primers were synthesized at Bioserve Biotechnologies (Laurel, MD). Advantage-HF Polymerase chain reaction (PCR) kit was from Clontech (Palo Alto, CA).

The pET based expression vector with ampR gene was used for construction of mutein clone. Plasmids were amplified in *Escherichia coli* (DH5 α high efficiency transformation) (GIBCO BRL Life Technology, Grand Island, NY) and DNA was extracted using Qiagen kits (Chatsworth, CA). TF-1 human erythroleukemia cell line was obtained from ATCC (Manassas, VA) and were grown in human granulocyte macrophage colony-stimulating factor. The PM-RCC renal cell carcinoma cell line was previously established (Obiri, N. I. et al., *J. Clin. Invest.* **91**:88-93 (1993)). THP-1 cells were kindly provided by Dr. Ray Donnelly (CBER FDA, Bethesda, MD). AIDS related Kaposi's sarcoma cell line KSY-1 cells was kindly provided by P. Gill (University of Southern California, Los Angeles, CA) (Husain, S. R. et al., *Nat. Med.* **5**:817-822 (1999)).

Construction of plasmids encoding IL-13R112D and *IL-13E13KR112D*

The mutagenesis of IL-13 gene was performed using a cDNA of wild type IL-13 (Minty, A. et al., *Nature* **362**:248-250 (1993)) as a template using sense primer *5'- taa ttt gcc cat atg tcc cca ggc cct gtg cct -3'*, anti-sense primer *5'- taa ttt gcc cga att cag ttg aag tct ccc tcg cg -3'* in order to mutate Arg112 to Asp (R112D) and incorporate NdeI and EcoRI restriction enzyme sites at 5'- and 3'- termini, respectively. Similarly, sense primer *5'- agg aga tat aca tat gtc ccc agg ccc tgt gcc tcc ctc tac agc cct cag gaa gct cat tga gga -3'* , and anti-sense primer primer *5'- taa ttt gcc cga att cag ttg aag tct ccc tcg cg -3'* were used to construct expression vector for *IL-13E13KR112D*. After subcloning the PCR products into pCR2.1® (Invitrogen, Carlsbad, CA), the plasmid was digested with NdeI and EcoRI. The fragment was inserted into an prokaryotic pET based expression vector digested with same restriction enzymes. The existence of mutation and restriction

sites was confirmed by sequencing of the plasmid.

Expression and purification of recombinant proteins

Expression and purification of wild type IL-13 and IL-13 mutants were carried out by essentially similar techniques as previously reported (Kreitman, R. J. et al., *Cytokine* **7**:311-318 (1995); Oshima, Y. et al., *J. Biol. Chem.* **275**:14375-14380 (2000)). The protein expression was induced by adding 1mM IPTG. WtIL-13 and all IL-13 mutants were produced in inclusion bodies. After washing, inclusion bodies were solubilized, refolded and purified by FPLC ion-exchange chromatography. The purified protein showed a prominent single band at approximately 13 kDa in Coomassie Blue stained SDS polyacrylamide gel (Fig. 1). IL-13PE38QQR fusion protein was expressed in *Escherichia coli* and purified as previously described (Debinski, W. et al., *J. Biol. Chem.* **270**:16775-16780 (1995)).

Cell proliferation assays:

Proliferation assays were performed as described previously (Oshima, Y. et al., *J. Biol. Chem.* **275**:14375-14380 (2000); Leland, P. et al., *Oncol. Res.* **7**:227-235 (1995)). Briefly, TF-1 cells were washed 2-3 times to remove GM-CSF and then $2X10^4$ cells per well were cultured in 96-well plates in RPMI medium containing 5 % fetal bovine serum. Varying concentration of wtIL-13, IL-13 mutants or both were added to the wells, and the cells were cultured for approximately 2 days. Tritiated thymidine (0.5 μCi) was added to each well 6-12 h before the plates were harvested in a Skatron cell harvester (Skatron, Inc., Sterling, VA). Glass fiber filter mats were counted in a beta plate counter (Wallac, Gaithersburg, MD).

Protein Synthesis Inhibition Assay

Protein synthesis inhibition assay was performed as previously described (Oshima, Y. et al., *J. Biol. Chem.* **275**:14375-14380 (2000); Puri, R. K. et al., *Cancer Res.* **51**:3011-3017 (1991)). In brief, $1X10^3$ PM-RCC cells were cultured with various concentrations of *IL-13PE38QQR* incubated for 20-24 h at 37 °C and then 1 μCi of ^{3}H-leucine (NEN, Boston, MA) was added to each well and cultured for an additional 4 h. For blocking experiments, 2 μg/ml of wild type IL-13 or IL-13 mutants was added prior to the addition of IL-13PE38QQR. Finally, cells were washed and harvested on

fiberglass filtermat and cell associated radioactivity was measured in a Beta Plate Counter (Wallac, Gaithersburg, MD). The concentration of *IL-13PE38QQR* at which 50 % inhibition of protein synthesis (IC_{50}) occurred was calculated.

IL-13 receptor binding studies

Recombinant human IL-13 was labeled with ^{125}I (Amersham) by using IODO-GEN reagent (Pierce, Rockford, IL) according to the manufacture's instructions. The specific activity of radiolabeled IL-13 was 18.0 - 26.4 μCi/μg. The equilibrium binding studies were carried out as described elsewhere (Obiri, N. I. et al., *J. Biol. Chem.* **270**:8797-8804 (1995); Obiri, N. I. et al., *J. Clin. Invest.* **91**:88-93 (1993), Oshima, Y. et al., *J. Biol. Chem.* **275**:14375-14380 (2000)). Briefly, $1X10^6$ cells in 100 μl binding buffer were incubated at 4 °C for 2 hr with ^{125}I-IL-13 (500 pM) in the absence or presence of various concentration of unlabeled wild type IL-13 or IL-13 mutein. Duplicate samples of cells associated with ^{125}I-IL-13 were separated from unbound ^{125}I-IL-13 by centrifugation through cushion of phthalate oils. The cell pellets were counted in a Gamma-counter (Wallac, Gaithersburg, MD).

Electrophoretic mobility-shift assay (EMSA)

EMSA was performed as described before (Murata, T. et al., *Int. J. Cancer* **70**:230-240 (1997); Murata, T. et al., *Blood* **91**:3884-3891 (1998); Oshima, Y. et al., *J. Biol. Chem.* **275**:14375-14380 (2000)). After incubation with various concentrations of wild type IL-13 or IL-13 mutants for 15 minutes, THP-1 cells, Tory Epstein-Barr virus (EBV) immortalized B cells (a gift from Dr. Giovanna Tosato, CBER/FDA), or KSY-1 cells were washed with cold PBS and solubilized with cold whole-cell extraction buffer (1 mM $MgCl_2$, 20 mM HEPES, pH 7.0, 10 mM KCl, 300 mM NaCl, 0.5 mM dithiothreitol, 0.1% NP-40, 1 mM PMSF, 1 mM Na_3VO_4 and 20% glycerol). DNA-protein interactions were assessed by EMSA using the Bandshift kit from Pharmacia. Briefly, 80 μg of sample proteins were incubated for 20 min. at room temperature with 1 ng of ^{32}P-labeled double stranded oligonucleotide probe (4.2 x 10^9 CPM/μg)SBE-1 (sense oligo *5'-gat cgc tct tct tcc cag gaa ctc aat g-3'*, anti sense oligo *5'-tcg aca ttg agt tcc tgg gaa gaa gag c-3'*) in binding buffer [10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 10% glycerol, 0.05% NP-40, 0.05 mg/ml poly $(dI\text{-}dC)_2$], and 2 μl of loading dye was added to samples which were then electrophoresed in a 5% non-reducing

binding of ^{125}I-IL-13. Double mutein IL-13 showed 1.3-3.0 times better affinity than wtIL-13. Introducing mutations did not alter the binding affinity dramatically.

IL-13 double mutein blocks proliferative activity of IL-13

Proliferative responses of wtIL-13, *IL-13R112D* and double mutein, *IL-13E13KR112D* was measured alone or in combinations of wtIL-13 and *IL-13E13KR112D* in TF-1 cell line which express type III IL-13R (composed of IL-13Rα1, IL-4Rα and IL-2Rγ chains). As expected, IL-13 stimulated the growth of TF-1 cells in a concentration-dependent manner. *IL-13R112D* was superior to wtIL-13 in stimulating TF-1 cell proliferation. In contrast, *IL-13E13KR112D* did not show any proliferative activity. To determine the effect of *IL-13E13KR112D* on IL-13 induced proliferation of TF-1 cells, cells were cultured in the presence of various concentration of *IL-13E13KR112D* and half maximal growth stimulatory concentration of wtIL-13. Interestingly, double mutein IL-13 significantly blocked the mitogenic activity of wtIL-13 in a concentration-dependent manner.

IL-13 double mutein can neutralize the downregulation of CD14 expression by wtIL-13 on monocytes:

Since IL-13 has been shown to downregulate CD14 expression on monocytes (Cosentino, G. et al., *J. Immunol.* **155**:3145-3151 (1995); Oshima, Y. et al., *J. Biol. Chem.* **275**:14375-14380 (2000)), we investigated whether IL-13 double mutein can nullify the downregulating activity induced by wtIL-13. wtIL-13 suppressed CD14 expression on monocytes and *IL-13E13KR112D* neutralized the effect of wtIL-13.

IL-13 double mutein blocks signal transduction induced by wtIL-13

STAT6 activation is early cellular response induced by IL-13 and is responsible for biological effect mediated by IL-13 including upregulation of MHC class II expression, CD23 expression, and switching of immunoglobulin class in B cells (Shimoda, K. et al., *Nature* **380**:630-633 (1996)). We therefore tested whether IL-13 double mutein can block the IL-13 induced signaling. Tory Epstein-Barr virus immortalized B cells and THP-1 monocytic cells express Type III IL-13 receptor while KSY-1 AIDS associated Kaposi's sarcoma cells express Type I IL-13 receptor. In all cell types both wtIL-13 and *IL-13R112D* induced STAT6 activation in a concentration

dependent manner, but *IL-13R112D* was 5-10 fold better than wtIL-13 in inducing STAT6 activation. In sharp contrast, double mutein IL-13 *IL-13E13KR112D* did not stimulate STAT6 activation even in the presence of very high concentrations. In addition, double mutein blocked the wtIL-13 induced STAT6 activation in THP-1 cell line. 10 ng/ml wtIL-13 stimulated maximal activation of STAT6, however in the presence of 10 fold excess of *IL-13E13KR112D* STAT6 activation was slightly suppressed. However, 50-fold excess of double mutein significantly blocked STAT6 activation.

IL-13E13KR112D blocks cytotoxicity mediated by IL-13PE38QQR.

A chimeric fusion protein composed of wtIL-13 and a mutated form of *Pseudomonas* exotoxin (PE38QQR) termed *IL-13PE38QQR* has previously been produced (e.g., Debinski, W. et al., *Clin. Cancer Res.* **1**:1253-1258 (1995); Debinski, W. et al., *J. Biol. Chem.* **270**:16775-16780 (1995); Puri, R. K. et al., *Blood* **87**:4333-4339 (1996); Debinski, W. et al., *J. Biol. Chem.* **271**:22428-22433 (1996); Husain, S. R. et al., *Clin. Cancer Res.* **3**:151-156 (1997); Maini, A. et al., *J. Urol.* **158**:948-953 (1997); Debinski, W. et al., *Nat. Biotechnol.* **16**:449-453 (1998); Husain, S. R. et al., *Blood* **95**:3506-3513 (2000)). This cytotoxin is highly cytotoxic to IL-13 receptor positive tumor cells *in vivo* and *in* vitro. *IL-13PE38QQR* mediates cytotoxicity through binding to IL-13 receptors and receptor internalization, therefore IL-13 receptor agonists and antagonists must be able to neutralize the cytotoxicity of the chimeric fusion toxin. To demonstrate interaction between IL-13 double mutein to IL-13 receptor, we tested whether *IL-13E13KR112D* can block the cytotoxicity mediated by *IL-13PE38QQR.*. *IL-13PE38QQR* mediated cytotoxicity in a concentration dependent manner, and IL-13, *IL-13R112D* or double mutein blocked this cytotoxicity in a concentration dependent manner in both cell types studied. *IL-13E13KR112D* seemed slightly superior than wtIL-13, however, it seemed inferior than *IL*-13R112D in the neutralization of *IL-13PE38QQR* induced cytotoxicity.

Example 18: Materials and Methods for Example 19

Materials

Sequence specific oligonucleotide primers were synthesized at Bioserve Biotechnologies (Laurel, MD). The pET based expression vector (Novagen, Madison, WI) was used for construction of mutein clone. Plasmids were amplified in *Escherichia*

coli, DH5α (GIBCO BRL Life Technology, Grand Island, NY) and DNA was extracted using plasmid purification kits (Qiagen, Chatsworth, CA). Restriction endonucleases and DNA ligase were obtained from New England Biolabs (Beverly, MA), BRL (Gaithersburg, MD), PANVERA (Madison, WI) and Boehringer Mannheim (Indianapolis, IN). The TF-1 human erythroleukemia cell line was purchased from ATCC (Manassas, VA). The PM-RCC renal cell carcinoma cell line was established in our laboratory (Obiri, N. I. et al., *J Clin Investig* **91**:88 (1993)). THP-1 , Ebstein-Barr Virus (EBV) immortalized B cell and AIDS-related Kaposi's sarcoma cell line KSY-1 were obtained and maintained as previously described (Oshima, Y. et al., *J Biol Chem* **275**:14375 (2000)).

Construction of plasmids encoding IL-13R112D and *IL-13E13K*

The mutagenesis of IL-13 gene was performed using cDNA of wtIL-13 (Minty, A. et al., *Nature* **362**:248 (1993)) as a template. Sense primer 5'- agg aga tat aca tat gtc ccc agg ccc tgt gcc tcc ctc tac agc cct cag gaa gct cat tga gga -3' , and anti-sense primer primer 5'- taa ttt gcc cga att cag ttg aac cgt ccc tcg cg -3' were used to mutate Glu 13 (E13) to Lys (K13) and incorporate NdeI and EcoRI restriction enzyme sites at the 5'- and 3'- termini, respectively. Construction of the expression vector for *IL-13R112D* was described before (Oshima, Y. et al., *J Biol Chem* **275**:14375 (2000)). After subcloning the PCR products, the fragment was restricted by NdeI and EcoRI and inserted into an expression vector. Existence of mutation and restriction sites was confirmed by sequencing of the plasmid.

Expression and purification of recombinant proteins

Expression and purification of wild type IL-13 and IL-13 mutants were carried out by techniques similar to those previously reported (Oshima, Y. et al., *J Biol Chem* **275**:14375 (2000); Kreitman, R. J. et al., *Cytokine* **7**:311 (1995)). WtIL-13 and IL-13 mutants were produced in inclusion bodies.

Cell proliferation assays

Proliferation assays were performed as described previously (Oshima, Y. et al., *J Biol Chem* **275**:14375 (2000); Leland, P. et al., *Oncology Research* **7**:227 (1995)). Briefly, $1X10^4$ TF-1 cells per well were cultured in 96-well plates in RPMI with 5% fetal

bovine serum. Varying concentrations of wtIL-13, IL-13 mutant or both were added to the wells, and the cells were cultured for approximately 2 days. Tritiated thymidine (0.5 μCi) was added to each well 6-12 h before the plates were harvested in a Skatron cell harvester (Skatron, Inc., Sterling, VA). Glass fiber filter mats were counted in a beta plate counter (Wallac, Gaithersburg, MD).

IL-13 receptor binding studies

WtIL-13 was labeled as previously described (Obiri, N. I. et al., *J Biol Chem* **270**:8797 (1995); Oshima, Y. et al., *J Biol Chem* **275**:14375 (2000)). The specific activity of radiolabeled IL-13 was 26 μCi/μg. The equilibrium binding studies were performed as described (Obiri, N. I. et al., *J Biol Chem* **270**:8797 (1995); Oshima, Y. et al., *J Biol Chem* **275**:14375 (2000)). Briefly, $5X10^5$ cells in 100 μl binding buffer were incubated at 4 °C for 2 hr with ^{125}I-IL-13 (200 or 500 pM) in the absence or presence of various concentrations of unlabeled wtIL-13 or IL-13 mutant. Receptor-bound ^{125}I-IL-13 was separated from unbound ^{125}I-IL-13. The cell pellets were counted in a Gamma-counter (Wallac, Gaithersburg, MD).

Electrophoretic mobility-shift assay (EMSA)

EMSA was performed as described before (Murata, T. et al., *International J Cancer* **70**:230 (1997); Murata, T. et al., *Blood* **91**:3884 (1998); Oshima, Y. et al., *J Biol Chem* **275**:14375 (2000)). After incubation with various concentrations of wtIL-13 or IL-13 mutants for 15 minutes, THP-1 cells, EBV immortalized B cells, or KSY-1 cells were washed with cold PBS and solubilized with cold whole-cell extraction buffer (1 mM $MgCl_2$, 20 mM HEPES, pH 7.0, 10 mM KCl, 300 mM NaCl, 0.5 mM dithiothreitol, 0.1% NP-40, 1 mM PMSF, 1 mM Na_3VO_4 and 20% glycerol). DNA-protein interactions were assessed by EMSA using the Bandshift kit (Pharmacia, Piscataway, NJ) using the ^{32}P-labeled double stranded oligonucleotide probe (4.2 x 10^9 CPM/μg) SBE-1.

CD14 regulation by IL-13

Primary monocytes were cultured at $1X10^7$ cells /ml for 48 h with 1 μg/ml wtIL-13 with or without 1 μg/ml *IL-13E13K*. Staining of the cells was performed as described elsewhere (Oshima, Y. et al., *J Biological Chemistry* **275**:14375 (2000)). The

fluorescence data were collected on a FACScan/C32 (Becton Dickinson, San Jose, CA). The results were analyzed with the CELLQuest (Becton Dickinson) program.

Sequence alignment and Molecular Modeling of IL-13Ra' CRH domain and IL-13

The sequences of CRH domains were aligned by the Bestfit program of GCG software (Genetics Computer Group, Inc., Madison, WI). Percent similarity and identity of extracellular domains between IL-13Ra' and IL-2Rg chains were 40.5% and 31.9 %, respectively. These numbers indicate reasonable sequence similarity justifying the use of IL-2Rγ chain as a template for modeling IL-13Rα' chain. Conserved sequence patterns such as "WSXWS" motif and disulfide bonds between β-strands of IL-13Rα' and IL-2Rγ were perfectly aligned. The alignment of sequences between hIL-4 and hIL-13 was also performed as previously reported (Minty, A. et al., *Nature* **362**:248 (1993); Bamborough, P. et al., *Protein Engineering* **7**:1077 (1994)). The similarity and identity of α-helix A and D of IL-13 to known structure of hIL-4 was in a similar range as observed for IL-2Rγ and IL-13Rα' chains. However, the similarity of α-helix B and C could not be reasonably aligned (Bamborough, P. et al., *Protein Engineering* **7**:1077 (1994)).

The coordinate of the CRH domain of the IL-4Rα chain was also used in our model and obtained from protein data bank entry 1ILL. The model building and refinement procedures in general followed the procedure previously described in detail (Greer, J. *Proteins* 7:317 (1990)). An initial model was built using the Homology module of InsightII (Molecular Simulations Inc., San Diego, CA). Small loops and splices were created and handled such that the energy was kept at minimum for best model. The structures were finally refined using the Discover program (Molecular Simulations Inc., San Diego, CA).

Example 19: IL-13E13K is an Antagonist of IL-13 Activity

Recombinant protein isolation and purification

Recombinant wtIL-13, *IL-13E13K and IL-13R112D* in which 112th arginine (R) residue of IL-13 molecule was substituted for aspartic acid (D) were expressed in *Escherichia coli* and purified from inclusion bodies as previously described (Oshima, Y. et al., *J Biol Chem* **275**:14375 (2000)). After purification, each recombinant

protein was analyzed using SDS-PAGE and stained with Coomassie blue. Each protein showed a prominent single band at approximately 13 kDa with purity of at least 95%.

IL-13E13K competes for the binding of radiolabeled IL-13

Binding studies were performed on U251 glioblastoma and PM-RCC renal cell carcinoma cell lines, both of which express type I IL-13 receptors (Obiri, N. I. et al., *J Immunol* **158**:756 (1997)). As expected, wtIL-13 displaced specific binding of radiolabeled IL-13. Interestingly, *IL-13E13K* also inhibited binding of ^{125}I-IL-13. *IL-13E13K* was better in displacing ^{125}I-IL-13 binding that wtIL-13. The EC_{50} (concentration causing 50% inhibition of ^{125}I-IL-13 binding) of wtIL-13 and *IL-13E13K* on U251 cells was approximately 20 nM and 2.5 nM, respectively. On PM-RCC, it was approximately 100 nM and 25 nM, respectively. Thus, *IL-13E13K* appeared to show approximately 4.0 to 8 fold better binding avidity than wtIL-13 in displacing ^{125}I-IL-13 binding.

IL-13E13K blocks proliferative activity of IL-13

TF-1 erythroleukemia cells proliferate in response to IL-13 (Oshima, Y. et al., *J Biol Chem* **275**:14375 (2000)). Proliferative activity of wtIL-13 and *IL-13E13K* was measured either alone or in combination of both. As expected, wtIL-13 stimulated the growth of TF-1 cells in a concentration-dependent manner (Oshima, Y. et al., *J of Biol Chem* **275**:14375 (2000)). In contrast, *IL-13E13K* did not show any proliferative activity. This result indicated that inserting a mutation at position 13 completely suppressed its agonistic activity and that the amino acid residue at position 13 seemed essential for the IL-13-induced proliferation of TF-1 cells. To determine the effect of *IL-13E13K* on wtIL-13 induced proliferation of TF-1 cells, we cultured cells in the presence of 1μg/ml *IL-13E13K* and various concentrations of wtIL-13. Interestingly, *IL-13E13K* blocked the mitogenic activity of wtIL-13. This block of IL-13 mitogenic activity was concentration-dependent. A 100 - 333 fold excess of *IL-13E13K* completely neutralized wtIL-13 induced mitogenic activity.

IL-13E13K can neutralize the downregulation of CD14 expression by wtIL-13 on human primary monocytes:

IL-13 has been shown to downregulate CD14 expression on monocytes (Cosentino, G. et al., *J Immunol* **155**:3145 (1995); Oshima, Y. et al., *J Biol Chem*

275:14375 (2000)). Therefore, we investigated whether *IL-13E13K* can nullify the downregulating activity induced by wtIL-13. wtIL-13 suppressed CD14 expression on monocytes and *IL-13E13K* completely neutralized the effect of wtIL-13. For example, IL-13 decreased the mean channel number (MCN or mean fluorescence intensity, MFI) in the gated region from 591 to 492 ($p < 0.01$). *IL-13E13K* reversed this effect and MCN recovered to 600.

IL-13E13K blocks signal transduction induced by wtIL-13

IL-13 has been shown to transduce signal through the Janus kinase (Jak) and signal transducer and activator of transcription (STAT) pathways (e.g., Murata, T. et al., *J Immunology* **156**:2972 (1996); Murata, T. et al., *Intl J Cancer* **70**:230 (1997); Murata, T. et al., *Intl Immunol* **10**:1103 (1998); Obiri, N. I. et al., *J Biol Chem* **272**:20251 (1997)). STAT6 is phosphorylated and activated after IL-13 stimulation, which in turn regulates gene transcription. We, therefore, tested whether *IL-13E13K* can block the IL-13 induced signaling. THP-1 and EBV immortalized B cells express Type II and Type III IL-13 receptors, respectively, while KSY-1 AIDS-associated Kaposi's sarcoma cells express Type I IL-13 receptors. In all cell types, both wtIL-13 and *IL-13R112D* induced STAT6 activation in a concentration dependent manner. *IL-13R112D*, a potent IL-13 agonist, also induced STAT6 activation and it was 5-10 fold better than wtIL-13 (Oshima, Y. et al., *J Biol Chem* **275**:14375 (2000)). In sharp contrast, *IL-13E13K* did not stimulate STAT6 activation even at very high concentrations (50 ng/ml). In addition, *IL-13E13K* blocked wtIL-13 induced STAT6 activation in the THP-1 cell line. 10 ng/ml wtIL-13 stimulated maximal activation of STAT6, however in the presence of 10-fold excess *IL-13E13K*, STAT6 activation was significantly suppressed and 50-fold excess of *IL-13E13K* almost completely blocked STAT6 activation.

Homology modeling of IL-13, IL-13Rα' and IL-4Rα

We created a model of interaction between the cytokine receptor homology (CRH) domains of IL-13Rα', IL-4Rα chains and IL-13 based on homology of CRH domains between IL-2Rγ and IL-13Rα' chains and between IL-4 and IL-13. This model was created based on our hypothesis that IL-13 interacts with IL-13Rα' and IL-4Rα chains simultaneously in the type II IL-13R complex. This model suggests that the

receptor binding interface of the IL-13 molecule is located in α-helix A and D, and that α-helix D and A may interact with IL-13α' and IL-4Rα chains, respectively.

Example 20: Materials and Methods Used in Example 21

Cells

L1236, L591 and L428 human Hodgkin's disease (HD)-derived cell lines have been established and well characterized (Diehl et al., *Cancer Surv.*, 4:399-419 (1985); Schaadt et al., *Intl J Cancer*; 26:723-31 (1980); Wolf et al., *Blood*; 87:3418-28 (1996)). TF-1 human erythroleukemia cells, A172 cells, and U251 human glioblastoma cells were obtained from the American Type Culture Collection (Manassas, VA). THP-1 human monocytic cells were kindly provided by Dr. Ray Donnelly (CBER, FDA, Bethesda, MD).

Antagonist, IL -13-toxin and IL-13 binding protein

IL-13 antagonist, *IL-13E13K* was produced as previously reported. IL-13-PE3SQQR was expressed and purified as previously reported (Puri et al., *Blood,* 87:4333-4339 (1996); Debinski et al., *Clin Cancer Research*; 1:1253-1258 (1995); Debinski et al., *J Biol Chem,* 270:16775-16780 (1995)). Recombinant human IL-13Rα2/Fc chimera, termed IL-13 binding protein (IL-13-BP) was purchased from R&D systems (Minneapolis, MN).

Rt-PCR

Rt-PCR analysis was performed essentially as previously reported (Murata et al., *Biochem Biophys Res Commun*; 238:90-94 (1997)). Total RNA was isolated with Sv-Total RNA Isolation System (Promega, Madison, WI). Rt-PCR was performed using Access RT-PCR System (Promega) according to manufacture's instruction. The primers used were: sense 5'-tcaacatcacccagaaccag-3' and antisense 5'-taagagcaggtcctttacaaac-3' for IL-13, sense 5'-ccatcattaccattcacatccc-3' and 5'-tctgttgttccagttcagttc-3' for IL-2Rγ, sense 5'-tggctttcgtttgcttgg -3' and antisense 5'-gcgtgtgtatcttcgcttc-3' for IL-13Rα2, sense 5'-accaatgagagtgagaagcc-3' and antisense 5'-tttcctgcattatccttgacc-3' for IL13Rαl, sense 5'-gctcttgccctgttttctg -3' and antisense 5'-tccctttttcttcctctacctc-3' for IL-4Rα and sense

5'-gtgggccgctctaggcacca-3' and antisense 5'-cggttggccttagggttcaggggg-3' for actin.

Determination of secreted IL-13 by HD cell line

L1236 and L428 cells were washed and 1.0 X 10^6 cells/ml were cultured for 72 hr. Culture supernatant was collected after centrifugation at 1,500 RPM for 5 minutes and stored at 70°C until assays. Dot blot analysis was performed using S&S Minifold®I (Schleicher & Schuell, Keene, NH) according to manufacturer's instructions. ELISA for IL-13 was performed using CYTELISA Human IL-13 kit (Cytimmune Sciences Inc., College Park, MD). Briefly, 1x10^6/ml cells were cultured for 3 days and the supernatant was harvested. 5 ml of supernatant was loaded on nytran® plus membrane (Schleicher & Schuell, Keene, NH) per well. The proteins were immobilized by incubating in 5% skim milk and then IL-13 was detected by incubating with 500 times diluted goat anti-human IL-13 polyclonal IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as a primary antibody and then incubated with 1000 x diluted anti-goat Ig-HRP conjugated (Santa Cruz Biotechnology) as a secondary antibody. The HRP activity was visualized by ECL™ (Amersham Life Science, Piscataway, NJ).

Cell proliferation and cytotoxicity assays

Cell proliferation (Leland et al., *Oncology Res*; 7:227-235 (1995)) and cytotoxicity assays (Puri et al., *Cancer Res,* 51:3011-3017 (1991)) were done essentially following the same techniques as previously reported. Studies of cell growth in the absence and in the presence of IL-13 (Figure 19, panel C) were performed as follows. 1x10^5/ml L1236, L591 or L428 cells were incubated with or without various concentrations of IL-13 for 54 hr in 37°C humidified 5% CO_2 incubator. After 1μCi per well ^{3}H-thymidine pulse per well, cells were incubated for an additional 12 hrs and then harvested by Skatron Cell Harvester (Skatron, Inc., Sterling, VA). Glass fiber filter mats were counted in a beta plate counter (Wallac, Gaithersburg, MD). Data in Figure 19, panel C is shown as mean ± standard deviation of percent CPM. Standard deviation is shown when bigger than symbol.

Studies of the cytotoxicity of an IL-13-targeted immunotoxin (Figure 19, panel D) were performed as follows. L1236 or L428 cells (1x10^4/ml)were incubated with or without various concentrations of IL-13-PE38QQR for 22 hr at 37°C in humidified 5% CO^2 incubator. Cells were pulsed with 1μCi per well ^{3}H-Leucine and incubated for an

additional 4 hr and then harvested and counted. Data is shown as mean ± standard deviation of percent CPM. Standard deviation is shown when bigger than symbol.

Example 21: IL-13 Antagonists Suppress Proliferation of Hodgkin's Disease Cells

Suppression of HD cell proliferation by IL-13 antagonist

The IL-13 antagonist, *IL-1 3E13K* discussed in Examples 18 and 19 was examined to determine whether this antagonist can suppress Hodgkin's disease/Reed-Sternberg ("H/RS" or "HD/RS") cell proliferation. The IL-13 antagonist significantly inhibited the proliferation of L1236 cells as determined by viable cell number counting or ^{3}H-thymidine uptake. In contrast, the growth of L428 cells was not affected by the antagonist, ruling out nonspecific toxicity of the antagonist.

Because equilibrated or secreted IL-13 in the culture media was washed out at the beginning of the assay, the effects of the antagonist were not so robust. Therefore, in order to show better dose response, HD/RS cells were cultured in the presence of wild-type IL-13 (0.1 ng/ml). This concentration was similar to the ED_{50} (concentration for half-maximum proliferation) of IL-13 on L1236 cells. Interestingly, this concentration was also similar to the IL-13 concentration in the culture media of L1236 cells determined by ELISA. IL-13 induced the proliferation of L1236 cells, which was also suppressed by IL-13 antagonist in a dose-dependent manner. The dose that induced half maximal inhibition of cell growth (IC_{50}) by the antagonist was approximately 20 ng/ml. For a positive control, we utilized an IL-13 binding protein ("BP") that suppresses IL-13 proliferative activity by absorbing IL-13. The IC_{50} of IL-13-BP (Zhang et al., *J Biol Chem*; 272:9474-9480 (1997)) on L1236 cells was also approximately 20 ng/ml. L428 cells were not affected by the antagonist or IL-13-BP in either assay.

Expression of IL-13 mRNA and functional receptor for IL-13

To determine the mechanism(s) of different activity of IL-13 and *IL-13E13K* on various HD/RS cell lines, we examined the expression of mRNA for IL-13R chains. All 3 HD/RS cell lines expressed mRNA for IL-13 and IL-13Rα1, IL-4Rα and IL-2Rγ chains. For a control, brain tumor cell lines U251 and A172 were examined. These cell lines were noted not to express IL-13 nor the IL-2Rγ chain, but to express IL-4Rα, IL-13Rα1 and IL-13Rα2 chains. These data show that brain tumor cell lines

express type I IL-13R. Hematopoietic cell lines TF-1 and THP-1 did not express IL-13 nor the IL-13Rα2 chain, but did express IL-4Rα, IL-13Rα1 and IL-2Rγ chains. Thus, these cell lines expressed type III IL-13R. These results also confirm our previous studies (Murata et al., *Biochem Biophys Res Commun*, 238:90-94 (1997)). Thus, all HD/RS cell lines tested expressed type III IL-13R, although a faint band for IL-13Rα2 chain was seen in L428 cells. All three HD/RS cell lines also expressed mRNA for IL-13 and all tested cell lines expressed protein for IL-13 as determined by dot blot analysis. The concentration of IL-13 determined by ELISA was 20 pg/ml and 187 pg/ml in L1236 and L428 cells, respectively.

The functional status of IL-13R was next investigated by two ways: 1) stimulation of proliferation by wild-type IL-13 and 2) internalization of IL-13 ligand-receptor complex. In the proliferation assay, L1236 cells responded to stimulation by IL-13, however, L428 and L591 cells did not. The ED_{50} of IL-13 in L1236 cells was approximately 0.1 ng/ml. Interestingly, the B9 murine B-cell line has a similar ED_{50} of IL-13. For internalization assay, a PE based recombinant toxin termed IL-13-PE3SQQR comprised of IL-13 and a mutated form of *Pseudomonas* exotoxin was utilized. This cytotoxin generally shows cytotoxicity via internalization of ligand-receptor complex (Puri et al., *Blood,* 87:4333-4339 (1996); Pastan et al., *Annual Rev Biochem,* 61:331-354 (1992)), therefore the cytotoxicity can be regarded as internalization. Interestingly, IL-13-PE38QQR was cytotoxic to L1236 cells but had no effect on L428 cells.

Example 22: Use of IL-13 Agonists in the Maturation of Dendritic Cells

Typically, peripheral blood derived monocytes are collected from normal donor or cancer patients. Monocytes are generally purified by standard elutriation density gradient technique or by plastic adherence for 2 hour at 37^0C. Elutriated or adherent cells are washed two times with HBSS (Life Technologies, Inc. Gaithersburg, MD) and resuspended in XVIVO-15 medium (BioWhittaker, Gaithersburg, MD) at a concentration of 1 $x10^6$ cell/ml. Three to five ml of this cell suspension is plated in each well of 6-well plate (Costar Corp., Cambridge, MA). The medium contains 100 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF) and 50 ng/ml of an IL-13R112D. After 7 days of culture at 37^0C in 5% CO_2, the culture medium is exchanged with fresh medium containing GM-CSF/IL-13R112D with TNF-alpha (10 ng/ml) and the cells are cultured for an additional 7 days. After 14-day of culturing, adherent cells (DCs) are loosened

gently using sterile cell scrapers. The cells are then transferred to sterile 50-ml centrifuge tubes and washed once and counted before phenotyping. These cells are now differentiated into dendritic cells, which acquire unique surface phenotypes. For example DCs begin to express CD11c, CD80, CD83 and high levels of HLA-DR but not CD14. The DCs are then pulsed as desired with an antigen, typically a tumor cell lysate, peptide antigen, apoptotic body fused to whole tumor cells, or a gene modified to express antigens and co-stimulatory molecules. Cells (one million to 10 million) are then washed with lactate ringer and injected to patients at multiple time points and schedules to boost their immune response to the antigen. The DCs migrate to lymph nodes and educate cytotoxic T cells in the context of MHC class I molecules. The T cells then circulate and seek and kill cells that express the antigen that was used to pulse DCs.

As little as 10 ng/ml IL-13, when combined with 10 or 100 ng/ml GM-CSF in the above protocol, can generate dendritic cells. Since IL-13R112D is up to 10 times more potent than wild type IL-13 in promoting the maturation and activation of potent DCs, as little as 0.1 ng/ml of IL-13R112D can be used in the above-described protocol to activate DCs. Thus, the agonists of the invention obviate the need for a high concentration of activator in the in vitro maturation of dendritic cells.

Example 23: Use of Antagonists of the Invention in Asthma, Allergic Rhinitis, and Atopic Dermatitis

Recent studies have demonstrated that IL-13 is a necessary and sufficient factor for the expression of allergic asthma. IL-13 also plays a key role in allergic rhinitis and atopic dermatitis. IL-13 induces pathophysiological features of asthma in animals in a manner that is independent of IgE, IL-4, and eosinophils (Wills-Karp et al., Science 282, 2258, 1998). Further studies in patients with asthma have demonstrated that IL-13 is locally produced in bronchoalveolar lavage (BAL) cells when these cells are challenged with allergen (Huang, et al. J. Immunol. 155:2688, (1995)). Up to 3 ng/ml of IL-13 protein/ml BAL was detected in allergen challenged patients. Therefore, neutralization of locally produced IL-13 will mitigate symptoms of this life-threatening disease.

The antagonists of IL-13 of the invention neutralize the effect of IL-13 and thus are useful in the treatment of asthma. To demonstrate this in an animal model of asthma, the following protocol may be utilized. Mice are typically sensitized to soluble antigens from the fungus *Aspergillus fumigatus* and subsequently challenged with an

intranasal bolus of spores from this fungus. The spore challenge in sensitized mice then sets in motion a chronic allergic lung disease characterized by airway hyperreactivity, mucus hypersecretion, smooth muscle hypertrophy and peribronchial fibrosis, all of which are features of clinical asthma (Kunkel et al., American J. Pathology 156:723-732 (2000)). The mice are first challenged intraperitoneally and subcutaneously with *aspergillus* antigen and then with fungus antigen given intratracheally. IL-13 antagonists (1 to 50 ng/ml diluted in 0.1% human serum albumin) are administered intratracheally several days after the aspergillus antigen challenge. IL-13 antagonists (1 to 50 microgram/kg, three times per week for two weeks) may also be given intraperitoneally or intravenously, for a total of 100 microliters of drug.

For the prevention of asthma in humans, IL-13 antagonists (1 to 50 micrograms/Kg) are administered three times per week for two weeks by injection or intranasally. Conveniently, the administration may be by inhaler or nebulizer.

Example 24: Prevention of Hepatic Fibrosis Induced by Schistosomiasis Infection

In Schistosomiasis, chronic parasite-induced granuloma formation can lead to tissue destruction and fibrosis, which causes much of the morbidity and mortality in humans. IL-13 has been shown to play a central role in the pathogenesis of schistosomiasis and appears to be a profibrotic agent (Chiarmonte et al., J. Clin. Invest. 104:777 (1999); Fallon et al., J. Immunol. 164:2585 (2000)). Moreover, fibrosis is a major pathological manifestation of a number of allergic, autoimmune and infectious diseases..

To demonstrate the activity of IL-13 antagonists in animal models, mice are infected with *Schistosoma mansoni* by percutaneous injection. Typically, these mice are acutely infected with this pathogen for 8 weeks (Smithers et al., Parasitology 55:695 (1965)). IL-13 antagonists are administered intraperitoneally or intravenously at a dose of 1 to 50 microgram/kg every alternate day for two weeks, and degree of fibrosis or resolution of fibrosis induced by infection is studied.

In clinical use, IL-13 antagonists can be administered i.p. or i.v. for the treatment of infection induced fibrosis. The starting dose of IL-13 antagonist is 1 microgram/kg, and can be escalated to 50 micrograms/kg. The doses will be given every alternate day for two weeks. In more intractable cases, a continuous infusion can be used, with a higher dose (100 microgram/Kg) of an IL-13 antagonist. For severe hepatic

fibrosis, portal infusion of an IL-13 antagonist (doses from 1 to 200 microgram/kg) may also be explored.

Example 25: Use of IL-13 Antagonists in the Slowing the Proliferation of Cancer Cells

IL-13 has been shown to be an autocrine growth factor for Hodgkin's' disease (HD) derived Reed Sternberg (RS) cells and renal cell carcinoma cell lines *in vitro*. IL-13 is also an autocrine growth factor for AIDS-associated Kaposi's sarcoma cells. It is expected that other cancer cell types also produce IL-13 and that IL-13 serves as an autocrine growth factor for these cancer cells as well. IL-13 antagonists will have a significant role in the management of cancers in which IL-13 acts as an autocrine growth factor.

The use of IL-13 antagonists in slowing or stopping the growth of cancer cells for which IL-13 acts as an autocrine growth factor can be demonstrated in animal models. In a typical protocol, up to 5 million HD/RS cells in 100-microliters of phosphate buffered saline are injected subcutaneously into nude Balb/C mice (male and female). After 4-6 days, when the mice have developed palpable tumors, they are injected with the IL-13 antagonist under study. Separate cohorts of mice are injected, with each cohort receiving a different dose of antagonist, with the doses ranging from 1 to 100 microgram/kg). The administrations can be intraperitoneal, intravenous, or intratumoral, and are given every alternate day for one to two weeks. Tumor response is determined by the measurement of tumor volume using Vernier calipers. Survival of the animals is followed. Additional cohorts of mice have the antagonist administered in a continuous infusion, at a higher dose (50 to 200 microgram/kg).

In the clinic, antagonists are administered in the same manner. If desired, and if the cancer is not of a type in which all cells are known to secrete IL-13, a test can be performed to determine whether proliferation of cells of the cancer will be slowed by administration of an IL-13 antagonist. For this test, a needle biopsy of tumor cells is obtained and the cells are tested for the production of IL-13 by standard ELISA tests, with the production of IL-13 being indicative that the patient will benefit from the administration of an IL-13 antagonist. This test can be confirmed by culturing the cells in the presence of an IL-13 antagonist and comparing their growth to a like culture of cells cultured in the absence of the antagonist.

Once it is confirmed that the tumor is an IL-13 secreting tumor or otherwise confirmed that IL-13 is an autocrine growth factor for the cancer, an IL-13 antagonist is administered. Typically, the starting dose of IL-13 antagonist is based on the amount of IL-13 produced by the cancer cells, with the goal being to contact the cells with a ten-fold or greater excess of inhibitor. Generally, one million cells produce up to 1 ng of IL-13 in three days. Assuming a tumor burden of $5x10^8$ tumor cells as an example, 5 microgram/kg would be administered as a starting dose every alternate day for two weeks. The doses can be escalated by half logs to 100 microgram/kg. Severe cases can also be treated by continuous infusion, commencing at a higher dose (100 microgram/kg) of antagonist.

Example 26: Use of IL-13 Antagonists in *Leishmania major* Infection

Leishmaniasis is an important disease affecting millions of people worldwide. Various mouse models are available that can be utilized for the study of immune response to disease. Using these model systems, it has been determined that IL-13 is a susceptibility factor for *Leishmania major* infection (Matthews et al., J. Immunol. 164:1458 (2000)). Blocking IL-13 can help shift the immune response to a Th1 type, that effects a resolution of the disease. High affinity-IL-13 antagonists can neutralize the effect of IL-13 in Leishmaniasis by systemic administration at the time of infection or after an infection has established.

Animal models are used to demonstrate the effect of IL-13 antagonists on this disease. IL-13 antagonists are administered to mice intraperitoneally or intravenously at the doses of 1 to 50 microgram/kg every alternate day for two weeks and the infection of the mice by Leishmania major induced infection studies.

In the clinic, patients are treated with an IL-13 antagonist in the clinic at 1 microgram/kg every alternate day for two weeks. If the response is not satisfactory in the judgment of the clinician, the dose of the antagonist is escalated by half logs to ~50 microgram/kg. Again, the doses are given every alternate day for two weeks. In severe cases, a continuous infusion can be administered, at a higher dose (100 microgram/Kg) of antagonist.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference for all purposes.

WHAT IS CLAIMED IS:

1. A method for preventing, or for reducing the severity of, a disease which is mediated by the activity of IL-13, said method comprising administering a mutated IL-13 which is an antagonist of IL-13 activity in an amount effective to prevent, or to reduce the severity of, said disease.

2. A method of claim 1, in which the mutated IL-13 in which a glutamic acid residue at position 13 is replaced by a neutrally charged or a positively charged amino acid residue.

3. A method of claim 2, wherein the glutamic acid residue at position 13 is replaced by an amino acid residue selected from a lysine and an arginine.

4. A method of claim 1, wherein the disease condition is selected from the group consisting of bronchial asthma, atopic dermatitis, allergic rhinitis, schistosomiasis, Leishmania, Hodgkin's disease, renal cell carcinoma, Kaposi's sarcoma and other cancers in which IL-13 serves as a growth factor.

5. The use of a mutated IL-13 in which a glutamic acid residue at position 13 of IL-13 is replaced by a neutrally charged or a positively charged amino acid residue for the manufacture of a medicament for the prevention or treatment of a disease which is mediated by the presence of IL-13.

6. The use of claim 5, wherein the disease is selected from the group consisting of bronchial asthma, atopic dermatitis, allergic rhinitis, schistosomiasis, Leishmania, Hodgkin's disease, renal cell carcinoma, Kaposi's sarcoma and other cancers in which IL-13 functions as a growth factor.

7. A method for augmenting an IL-13-mediated response in a cell, said method comprising contacting said cell with a mutated IL-13, which mutated IL-13 has one or more mutations selected from the group consisting of replacing an arginine residue at position 112 of IL-13 by a neutrally charged or a negatively charged amino acid residue, replacing a glutamic acid residue at position 110 of IL-13 with a neutrally charged or a positively charged residue, replacing an arginine at position 109 of IL-13 with a neutrally charged or a negatively charged residue, replacing a glutamic acid

residue at position 92 of IL-13 with a neutrally charged or a positively charged residue, replacing a positively charged residue at position 69 with a neutrally charged or a negatively charged residue, and replacing positively charged residue at position 66 with a neutrally charged or a negatively charged residue, with the proviso that a position of said mutated IL-13 corresponding to position 13 of wild-type IL-13 is not occupied by a lysine residue.

8. A method of claim 7, wherein the arginine at position 112 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid.

9. A method of claim 7, wherein the glutamic acid at position 110 of IL-13 is replaced by a residue selected from the group consisting of lysine and arginine.

10. A method of claim 7, wherein the arginine at position 109 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid.

11. A method of claim 7, wherein the glutamic acid position 92 of IL-13 is replaced by a residue selected from the group consisting of lysine and arginine.

12. A method of claim 7, wherein a positively charged residue at position 69 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid.

13. A method of claim 7, wherein a positively charged residue at position 66 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid.

14. A method of claim 7, wherein said contacting of said cell occurs *in vitro*.

15. A method of claim 7, wherein said contacting of said cell occurs *in vivo*.

16. A use of a mutated IL-13 for the manufacture of a medicament for pretreating bone marrow stem cell donors, which mutated IL-13 has one or more

mutations selected from the group consisting of replacing an arginine residue at position 112 of IL-13 by a neutrally charged or a negatively charged amino acid residue, replacing a glutamic acid residue at position 110 of IL-13 with a neutrally charged or a positively charged residue, replacing an arginine at position 109 of IL-13 with a neutrally charged or a negatively charged residue, replacing a glutamic acid residue at position 92 of IL-13 with a neutrally charged or a positively charged residue, replacing a positively charged residue at position 69 with a neutrally charged or a negatively charged residue, and replacing positively charged residue at position 66 with a neutrally charged or a negatively charged residue; with the proviso that a position of said mutated IL-13 corresponding to position 13 of wild-type IL-13 is not occupied by a lysine residue.

17. A use of claim 16, wherein the arginine at position 112 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid.

18. A use of claim 16, wherein the glutamic acid at position 110 of IL-13 is replaced by a residue selected from the group consisting of lysine and arginine.

19. A use of claim 16, wherein the arginine at position 109 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid.

20. A use of claim 16, wherein the glutamic acid position 92 of IL-13 is replaced by a residue selected from the group consisting of lysine and arginine.

21. A use of claim 16, wherein a positively charged residue at position 69 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid.

22. A use of claim 16, wherein the arginine at position 66 of IL-13 is replaced by a residue selected from the group consisting of glutamic acid and aspartic acid.

23. An IL-13-receptor binding molecule selected from an IL-13, a circularly permuted IL-13, and a molecule with at least about 85% identity to IL-13, which IL-13-receptor binding molecule

(a) has a binding affinity for an IL-13 receptor at least about three times higher than that of wild-type IL-13, and

(b) comprises one or more mutations selected from the group consisting of changing

(i) an amino acid corresponding to a glutamic acid at position 110 of IL-13 to a neutrally charged or to a positively charged amino acid,

(ii) an amino acid corresponding to a glutamic acid at position 92 of IL-13 to a neutrally charged or to a positively charged amino acid.

24. An IL-13-receptor binding molecule of claim 23 wherein said molecule is an IL-13 or a circularly permuted IL-13.

25. An IL-13-receptor binding molecule of claim 23, wherein the glutamic acid at position 110 of IL-13 is replaced by a lysine or an arginine.

26. An IL-13-receptor binding molecule of claim 23, wherein the glutamic acid at position 92 of IL-13 is replaced by a lysine or an arginine.

27. An IL-13-receptor binding molecule of claim 23, wherein the glutamic acid at position 92 of IL-13 and the glutamic acid at position 110 of IL-13 are replaced, respectively, by a residue independently selected from the group consisting of lysine and arginine.

28. An IL-13-receptor binding molecule of claim 23, wherein the IL-13 or circularly permuted IL-13 has proliferative activity at least about twice that of wild-type IL-13 as measured in TF-1 cells.

29. A chimeric molecule that specifically binds a tumor cell bearing an IL-13 receptor, said chimeric molecule comprising an IL-13-receptor binding molecule of claim 23, and an effector molecule.

30. A chimeric molecule of claim 29, wherein said IL-13-receptor binding molecule is an IL-13 or a circularly permuted IL-13.

31. A chimeric molecule of claim 29, wherein said effector molecule is selected from the group consisting of a cytotoxin, a label, a radionuclide, a drug, a liposome, a ligand, and an antibody.

32. A chimeric molecule of claim 29, wherein said cytotoxin is selected from the group consisting of a *Pseudomonas* exotoxin or cytotoxic fragment thereof, *Diphtheria* toxin or cytotoxic fragment thereof, ricin or cytotoxic fragment thereof, saponin, gelonin, ribosome inactivating protein, and abrin.

33. A chimeric molecule of claim 29, wherein said *Pseudomonas* exotoxin is selected from the group consisting of PE38, PE3QQR, PE38KDEL, and PE4E.

34. A chimeric molecule of claim 29, wherein said molecule is a single chain fusion protein.

35. A composition comprising a pharmaceutically acceptable carrier and a chimeric molecule, said chimeric molecule comprising an IL-13-receptor binding molecule of claim 23, attached to an effector molecule.

36. The composition of claim 35, wherein said IL-13-receptor binding molecule is an IL-13 or a circularly permuted IL-13.

37. The composition of claim 35, wherein said chimeric molecule is a single-chain fusion protein.

38. The composition of claim 35, wherein said effector molecule is selected from the group consisting of a cytotoxin, a label, a radionuclide, a drug, a liposome, a ligand, and an antibody.

39. The composition of claim 38, wherein said cytotoxin is a modified *Pseudomonas* exotoxin A or cytotoxic fragment thereof.

40. The composition of claim 39, wherein said *Pseudomonas* exotoxin is selected from the group consisting of PE38, PE38QQR, PE38KDEL, and PE4E.

41. A method for specifically delivering an effector molecule to a tumor cell bearing an IL-13 receptor, said method comprising:

providing a chimeric molecule comprising said effector molecule attached to an IL-13-receptor binding molecule with a binding affinity for an IL-13 receptor at

least about three fold higher than that of wild-type IL-13, which IL-13-receptor binding molecule is at least about 85% identical to IL-13 and which comprises one or more mutations selected from the group consisting of changing

(i) an amino acid corresponding to a glutamic acid at position 110 of IL-13 to a neutrally charge or to a positively charged amino acid, and

(ii) an amino acid corresponding to a glutamic acid at position 92 of IL-13 to a neutrally charged or to a positively charged amino acid; and

contacting said tumor with said chimeric molecule;

wherein said chimeric molecule specifically binds to an IL-13 receptor on said tumor cell.

42. The method of claim 41, wherein said IL-13 -receptor binding molecule is an IL-13 or a circularly permuted IL-13.

43. The method of claim 41, wherein said tumor cell is selected from the group consisting of a carcinoma cell and a sarcoma cell.

44. The method of claim 43, wherein said tumor cell is selected from the group consisting of a renal cell carcinoma cell, a glioma cell, a medulloblastoma cell, a head and neck cancer cell, a pancreatic cancer cell, and a Kaposi's sarcoma cell.

45. The method of claim 41, wherein said effector molecule is selected from the group consisting of a cytotoxin, a label, a radionuclide, a drug, a liposome, a ligand, and an antibody.

46. The method of claim 41, wherein said effector molecule is a cytotoxin.

47. The method of claim 46, wherein said cytotoxin is selected from the group consisting of *Pseudomonas* exotoxin or cytotoxic fragment thereof, *Diphtheria* toxin or a cytotoxic fragment thereof, ricin or a cytotoxic fragment thereof, saponin, gelonin, ribosome inactivating protein, and abrin.

48. The method of claim 47, wherein said *Pseudomonas* exotoxin is selected from the group consisting of PE4E, PE38, PE38QQR, and PE38KDEL.

49. The method of claim 41, wherein said chimeric molecule is a fusion protein.

50. The method of claim 49, wherein said fusion protein is IL-13-PE38, wherein position 110 or position 92 of the IL-13 moiety is an amino acid residue selected from the group consisting of an aspartic acid and a glutamic acid.

51. The method of claim 49, wherein said fusion protein is cpIL-13-PE38, wherein position 110 or position 92 of the cpIL-13 moiety is an amino acid residue selected from the group consisting of an aspartic acid and a glutamic acid.

52. A method for impairing growth of a tumor cell bearing an IL-13 receptor, said method comprising contacting said tumor cell with a chimeric molecule comprising:

an IL-13-receptor binding molecule with a binding affinity for an IL-13 receptor at least about three fold higher than that of wild-type IL-13, which IL-13-receptor binding molecule is at least about 85% identical to IL-13 and which comprises one or more mutations selected from the group consisting of changing

(i) an amino acid corresponding to a glutamic acid at position 110 of IL-13 to a neutrally charged or to a positively charged amino acid, and

(ii) an amino acid corresponding to a glutamic acid at position 92 of IL-13 to a neutrally charged or to a positively charged amino acid; and

an effector molecule selected from the group consisting of a cytotoxin, a radionuclide, a ligand and an antibody;

wherein said effector molecule inhibits the growth of a tumor cell in contact with said effector molecule.

53. The method of claim 52, wherein said chimeric molecule is a single-chain fusion protein

54. The method of claim 52, wherein said effector molecule is a cytotoxin.

55. The method of claim 54, wherein said cytotoxin is selected from the group consisting of *Pseudomonas* exotoxin A or a cytotoxic fragment thereof, ricin or

a cytotoxic fragment thereof, abrin, saponin, gelonin, ribosome inactivating protein, and *Diphtheria* toxin or a cytotoxic fragment thereof.

56. The method of claim 55, wherein said cytotoxin is a modified *Pseudomonas* exotoxin A or cytotoxic fragment thereof.

57. The method of claim 52, wherein said tumor cell growth is in a human.

58. The method of claim 52, wherein said contacting comprises administering said chimeric molecule to the human into a vein, into a body cavity, into a lumen, or into an organ.

59. A method for detecting the presence or absence of a tumor, said method comprising contacting said tumor with a chimeric molecule comprising:

an IL-13-receptor binding molecule with a binding affinity for an IL-13 receptor at least about three fold higher than that of wild-type IL-13, which IL-13-receptor binding molecule comprises one or more mutations selected from the group consisting of changing

(i) an amino acid corresponding to a glutamic acid at position 110 of IL-13 to a neutrally charged or to a positively charged amino acid, and

(ii) an amino acid corresponding to a glutamic acid at position 92 of IL-13 to a neutrally charged or to a positively charged amino acid;

and a detectable label; and

detecting the presence or absence of said label.

60. A vector comprising a nucleic acid sequence encoding a chimeric fusion protein comprising an IL-13-receptor binding molecule with a binding affinity for an IL-13 receptor at least about three fold higher than that of wild-type IL-13, which IL-13-binding receptor molecule is at least about 85% identical to IL-13 and which comprises one or more mutations selected from the group consisting of changing

(i) an amino acid corresponding to a glutamic acid at position 110 of IL-13 to a neutrally charged or to a positively charged amino acid, and

(ii) an amino acid corresponding to a glutamic acid at position 92 of IL-13 to a neutrally charged or to a positively charged amino acid,

wherein said chimeric fusion protein specifically binds to a tumor cell bearing an IL-13 receptor.

61. The vector of claim 60, wherein said nucleic acid sequence encodes an IL-13-PE fusion protein.

62. The vector of claim 60, wherein said nucleic acid sequence encodes a circularly permuted IL-13-PE fusion protein.

63. A host cell comprising a nucleic acid sequence encoding a chimeric fusion protein comprising an IL-13-receptor binding molecule with a binding affinity for an IL-13 receptor at least about three fold higher than that of wild-type IL-13, which IL-13-receptor binding molecule is at least about 85% identical to IL-13 and comprises one or more mutations selected from the group consisting of changing

(i) an amino acid corresponding to a glutamic acid at position 110 of IL-13 to a neutrally charged or to a positively charged amino acid,

(ii) an amino acid corresponding to a glutamic acid at position 92 of IL-13 to a neutrally charged or to a positively charged amino acid,

and wherein said chimeric fusion protein specifically binds to a tumor cell bearing an IL-13 receptor.

64. The host cell of claim 63, wherein said nucleic acid sequence encodes an IL-13-PE fusion protein.

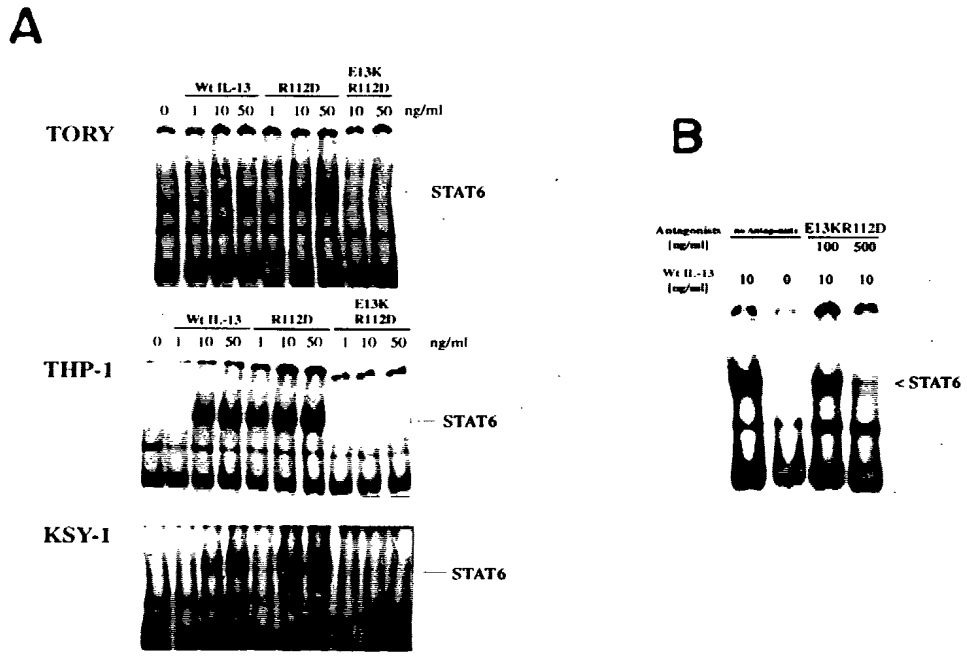

A
Wt IL-13
R112D
E13K R112D
0 1 10 50 1 10 50 10 50 ng/ml
TORY
STAT6
THP-1
KSY-1
B
E13KR112D
100 500
10 0 10 10
< STAT6

FIG. 10.

```
                1                                                  50
      MOUSE APGPVPRSVS LPLTLKELIE ELSNITQDQ. TPL NGSMVW SVDLAAGGF
        RAT TPGPVRRSTS PPVALRELIE ELSNITQDQK TSL NSSMVW SVDLTAGGF
      HUMAN SPGPVPPST. ...ALRELIE ELVNITQNQK APL NGSMVW SINLTAGMY
BOS TAURUS SPSPVPSAT. ...ALKELIE ELVNITQNQK VPL NGSMVW SLNLTSSMY

               51                                                 100
      MOUSE VALDSLTNIS N NAIYRTQR ILHGL NRK. .APTTVSS.. LPDTKIEVA.
        RAT AALESLTNIS S NAIHRTQR ILNGL NQK. .ASDVASS.. PPDTKIEVAQ
      HUMAN AALESLINVS G SAIEKTQR MLGGF PHKV SAGQ.FSSLH VRDTKIEVAQ
BOS TAURUS AALDSLISIS N SVIQRTKK MLNAL PHKP SAKQ.VSSEY VRDT.IEVAQ

              101
      MOUSE HFITK.LLSY TKQLFRH.GP F
        RAT .FISK.LLNY SKQLRF.YGH
      HUMAN .FV.KDLLLH LKKLFR.EGR FN
BOS TAURUS KFL.KDLLRH SRIVFRNE.R FN
```

FIG. 1.

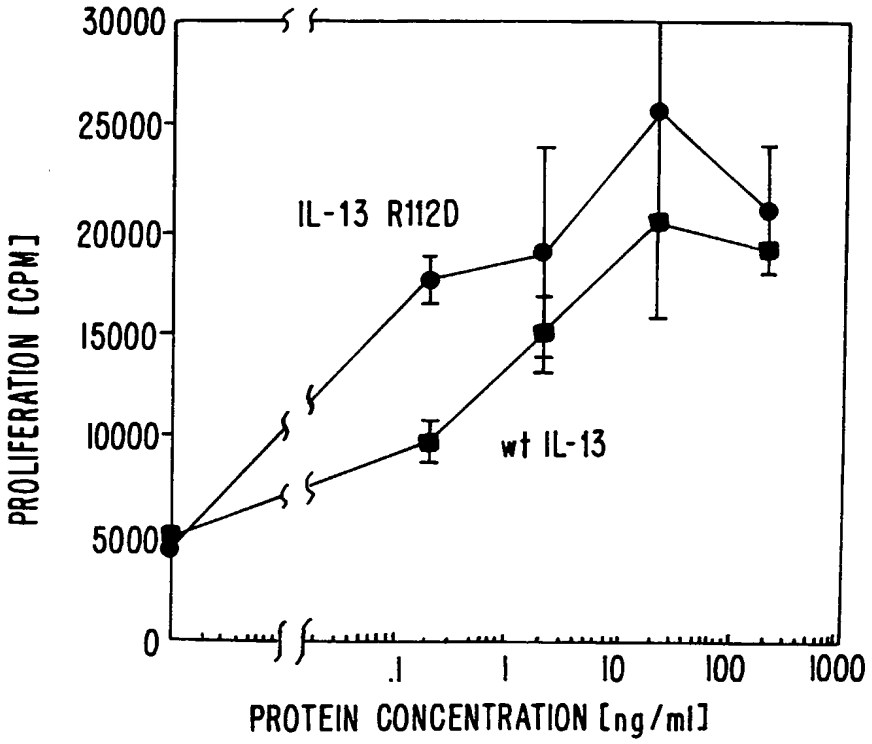

30000
25000
20000
15000
10000
5000
0
PROLIFERATION [CPM]
IL-13 R112D
wt IL-13
.1
1
10
100
1000
PROTEIN CONCENTRATION [ng/ml]

FIG. 2A.

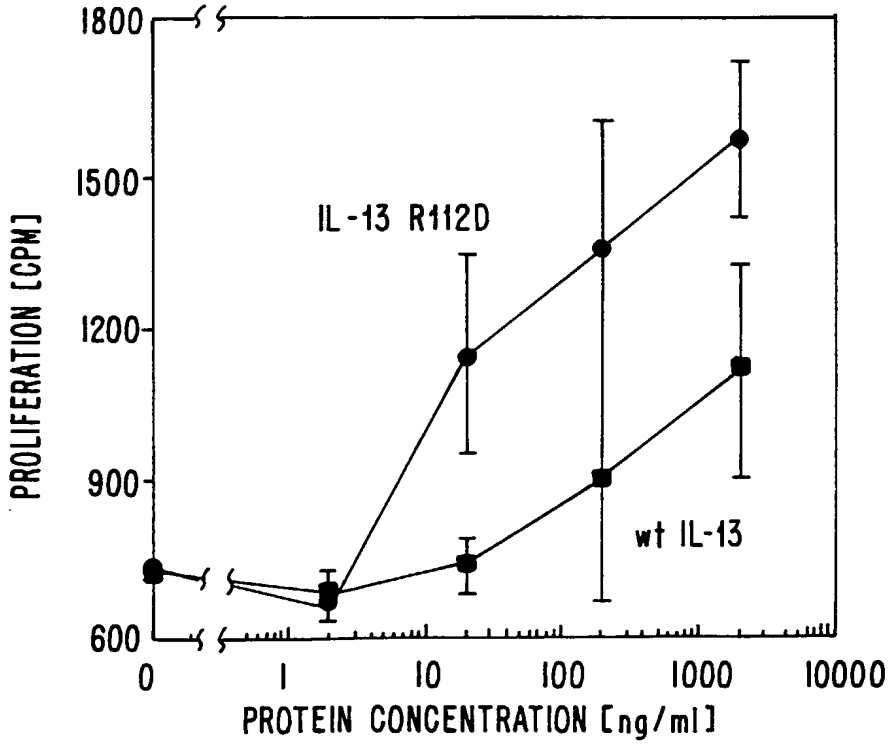

1800
1500
1200
900
600
PROLIFERATION [CPM]
IL-13 R112D
wt IL-13
0
1
10
100
1000
10000
PROTEIN CONCENTRATION [ng/ml]

FIG. 2B.

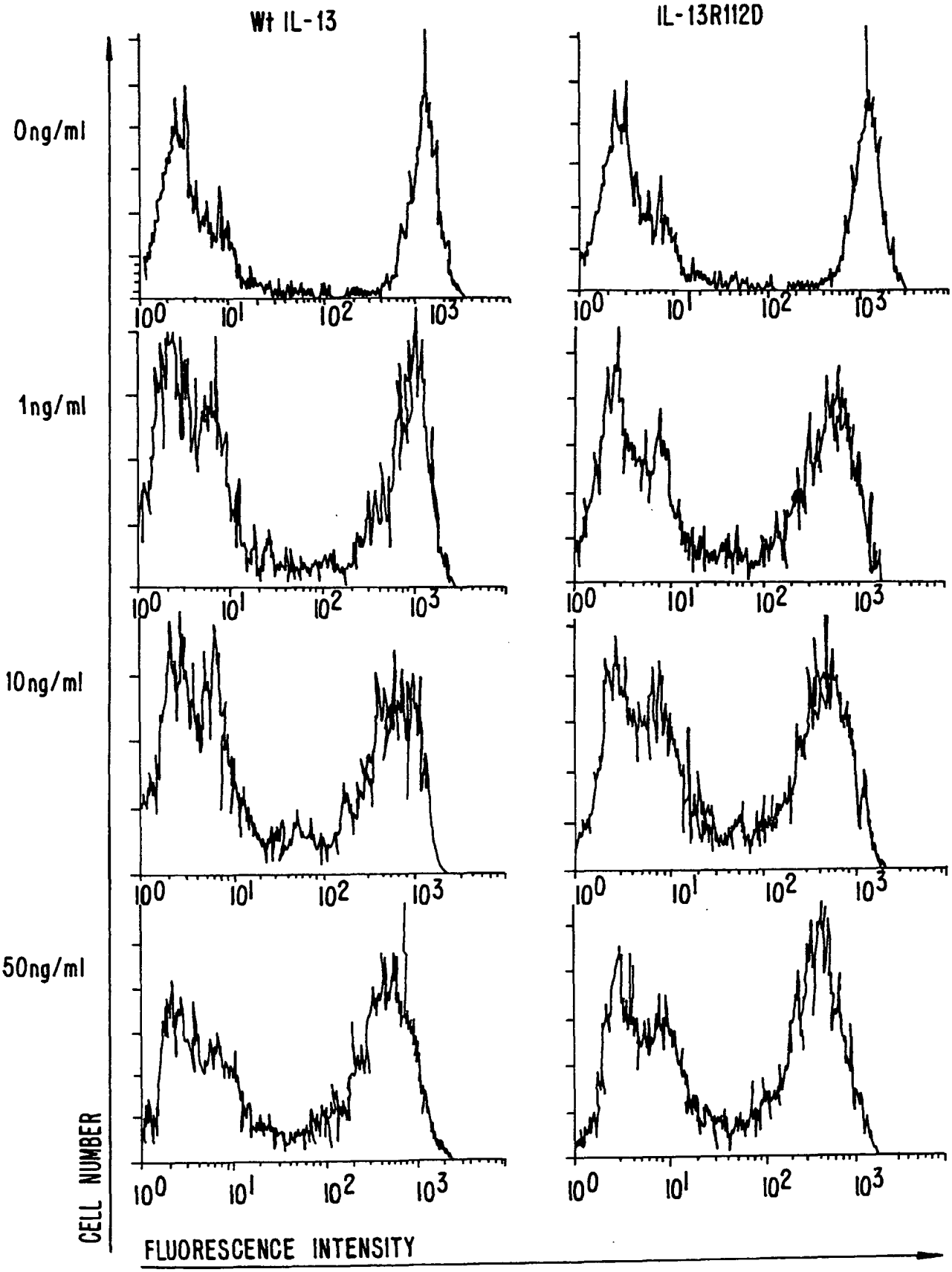

Wt IL-13
IL-13R112D
0ng/ml
1ng/ml
10ng/ml
50ng/ml
10^0 10^1 10^2 10^3
CELL NUMBER
FLUORESCENCE INTENSITY

FIG. 3A.

IL-13 R112D	0 ng/ml	1 ng/ml	10 ng/ml	50 ng/ml
Mean Fluorescent Intensities	1113.97	457.25	378.55	358.66

wt IL-13	0 ng/ml	1 ng/ml	10 ng/ml	50 ng/ml
Mean Fluorescent Intensities	1113.97	723.39	465.55	395.96

FIG. 3B.

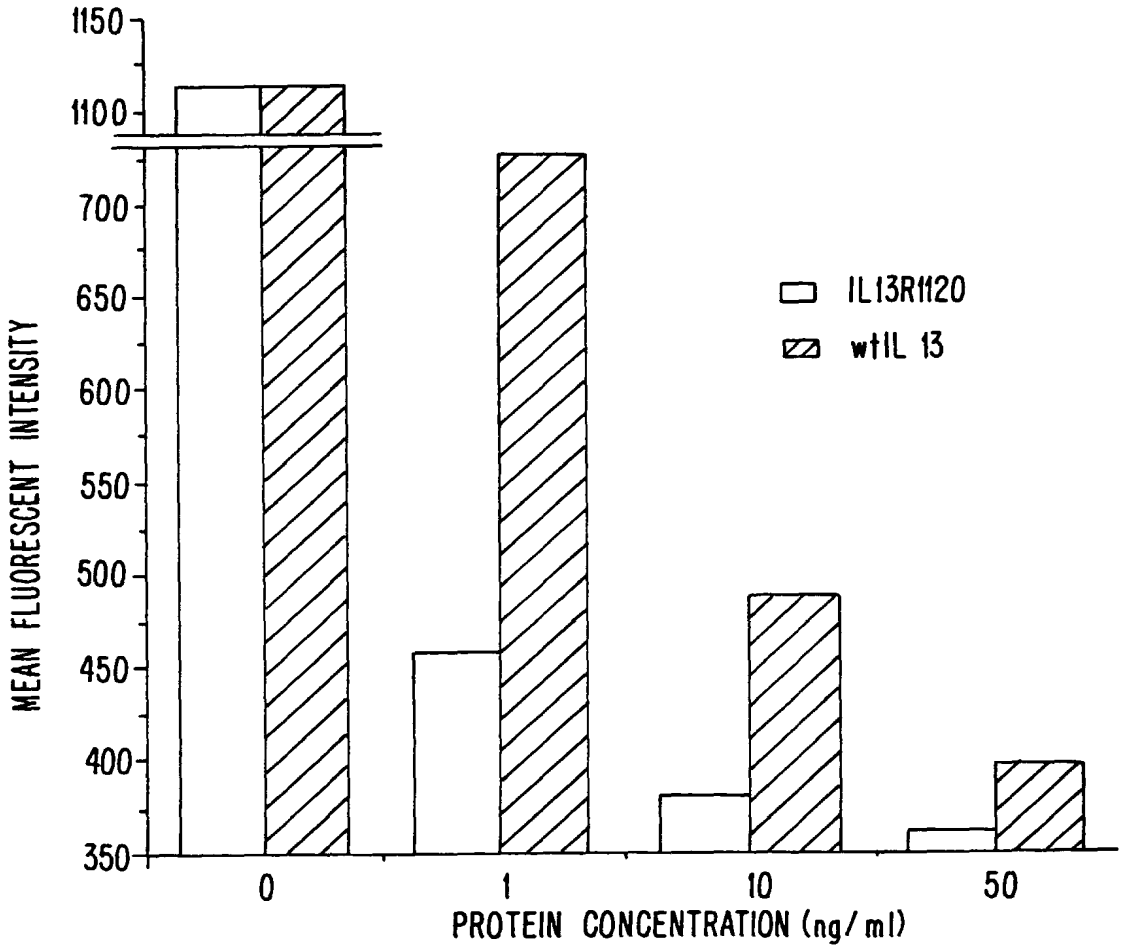


FIG. 3C.

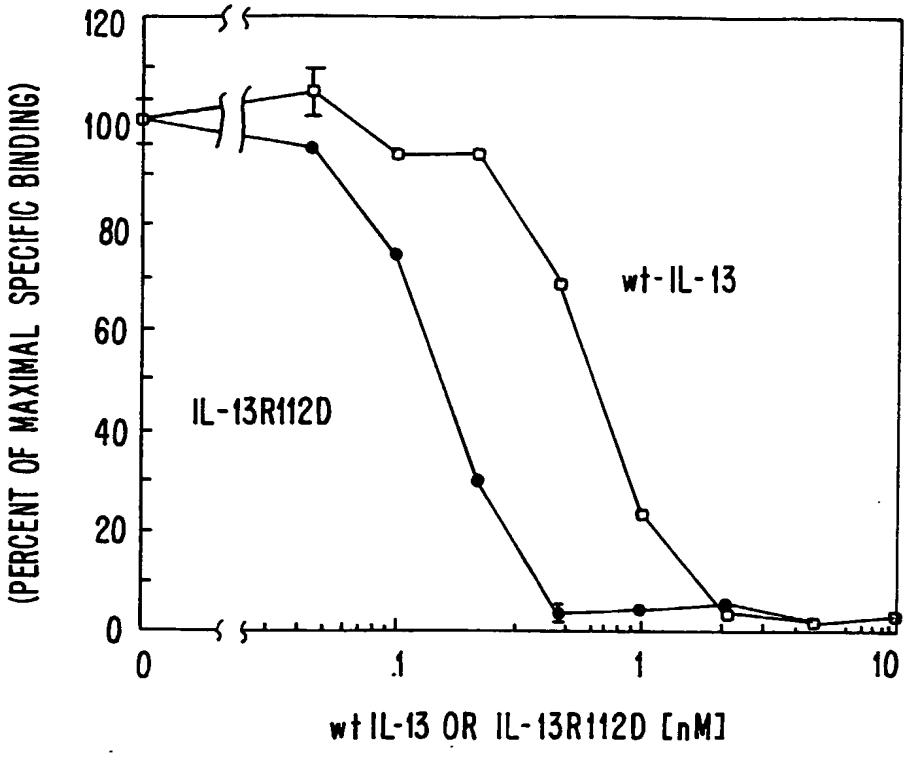

120
100
80
60
40
20
0
(PERCENT OF MAXIMAL SPECIFIC BINDING)
wt-IL-13
IL-13R112D
0
.1
1
10
wt IL-13 OR IL-13R112D [nM]

FIG. 4A.

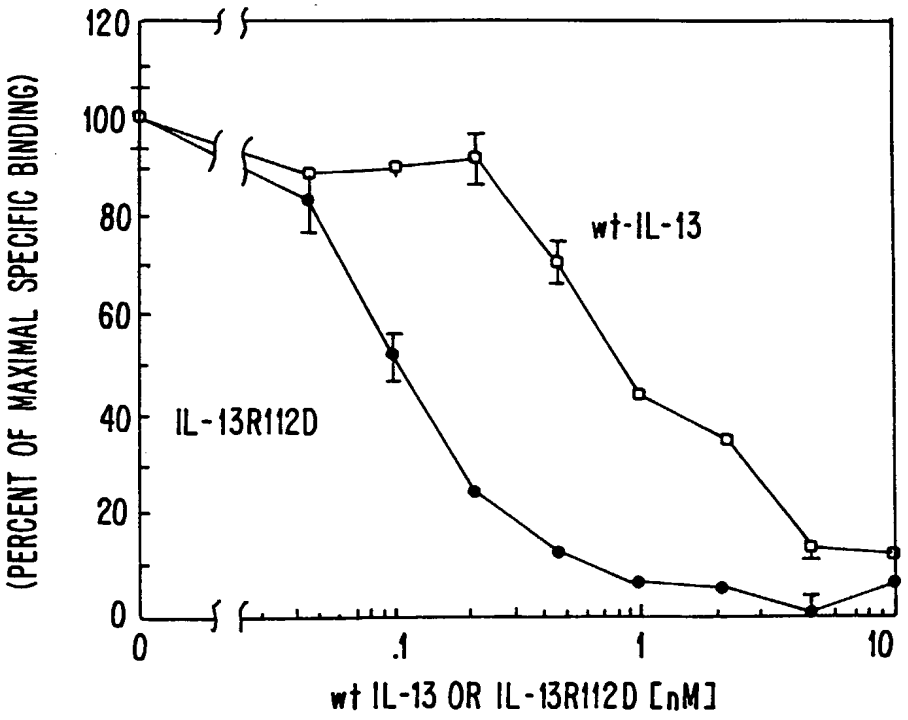

120
100
80
60
40
20
0
(PERCENT OF MAXIMAL SPECIFIC BINDING)
wt-IL-13
IL-13R112D
0
.1
1
10
wt IL-13 OR IL-13R112D [nM]

FIG. 4B.

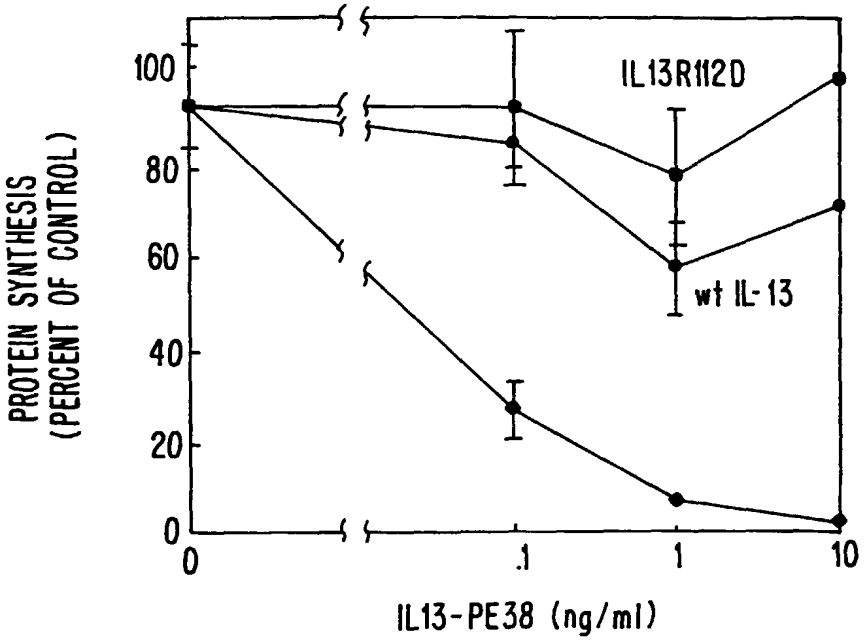

PROTEIN SYNTHESIS
(PERCENT OF CONTROL)
100
80
60
40
20
0
0
.1
1
10
IL13R112D
wt IL-13
IL13-PE38 (ng/ml)

FIG. 5A.

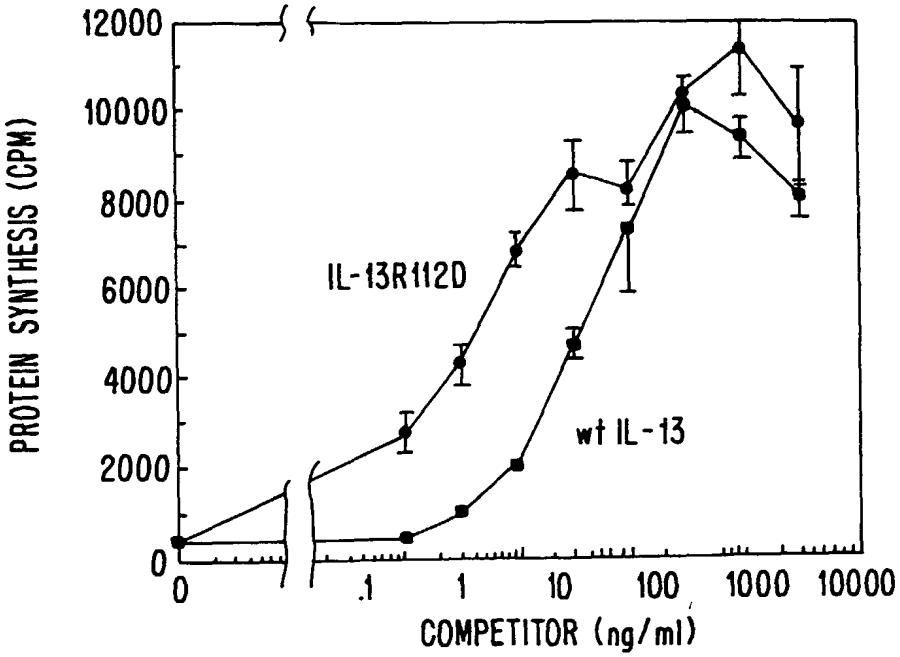

PROTEIN SYNTHESIS (CPM)
12000
10000
8000
6000
4000
2000
0
0
.1
1
10
100
1000
10000
IL-13R112D
wt IL-13
COMPETITOR (ng/ml)

FIG. 5B.

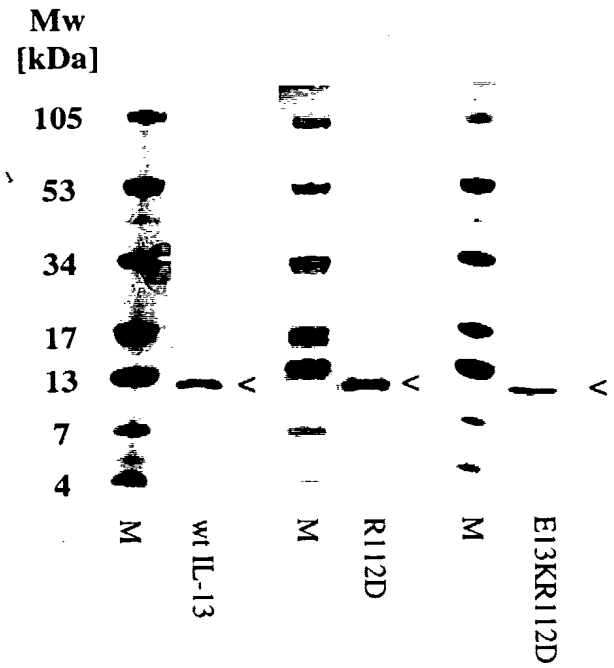


FIG. 6.

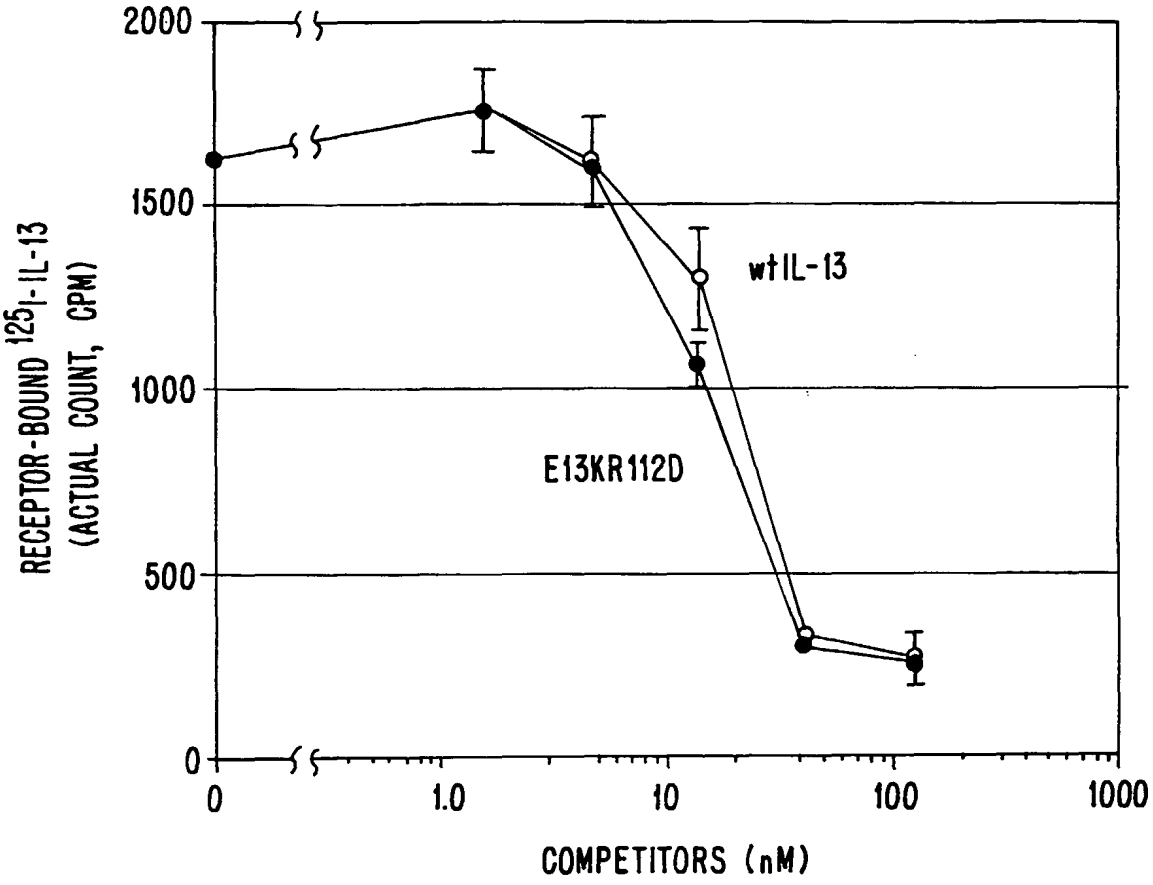

2000
1500
1000
500
0
RECEPTOR-BOUND ^{125}I-IL-13
(ACTUAL COUNT, CPM)
wtIL-13
E13KR112D
0
1.0
10
100
1000
COMPETITORS (nM)

FIG. 7.

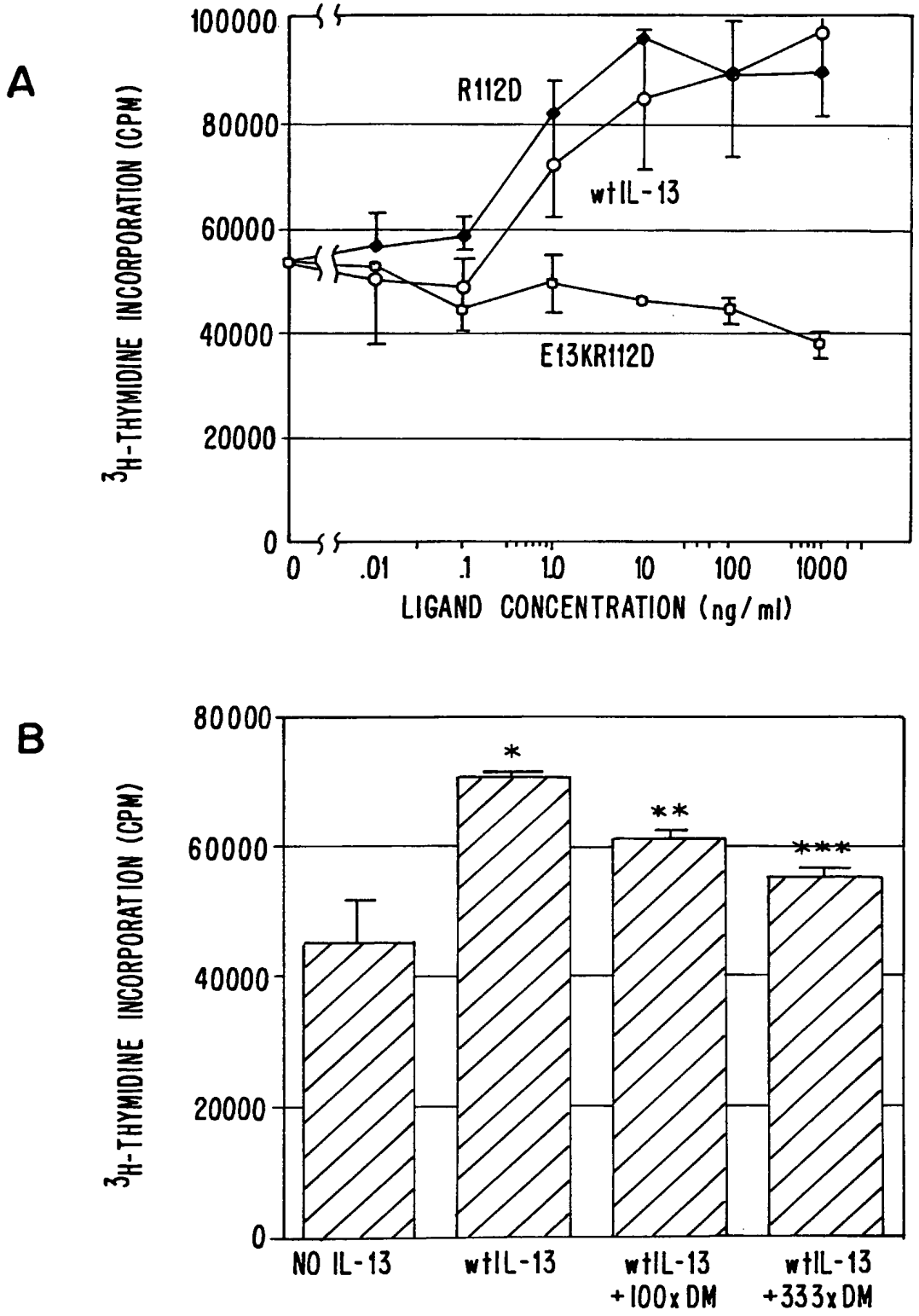
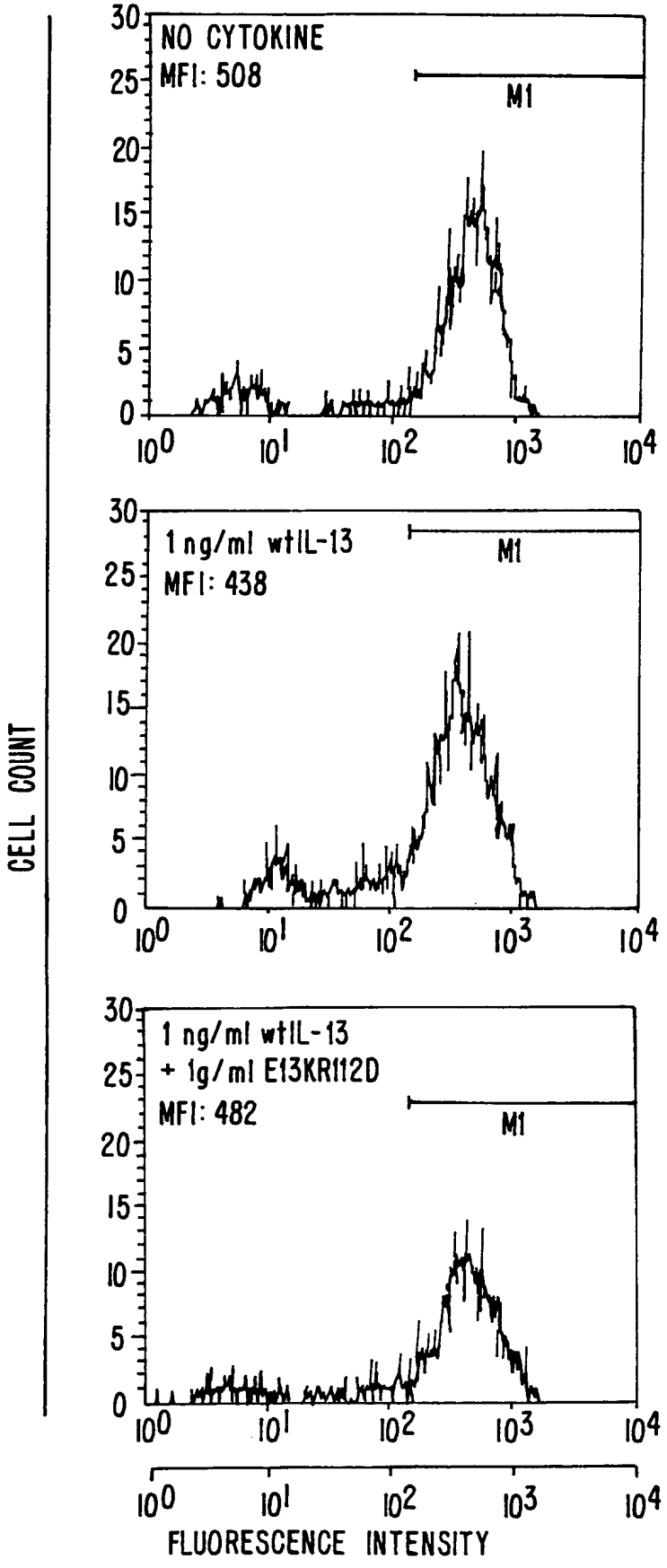

A
100000
80000
60000
40000
20000
0
R112D
wtIL-13
E13KR112D
^{3}H-THYMIDINE INCORPORATION (CPM)
0
.01
.1
1.0
10
100
1000
LIGAND CONCENTRATION (ng/ml)
B
80000
60000
40000
20000
0
*
**
***
^{3}H-THYMIDINE INCORPORATION (CPM)
NO IL-13
wtIL-13
wtIL-13 +100x DM
wtIL-13 +333x DM

FIG. 8.

FIG. 9.


NO CYTOKINE
MFI: 508
M1
1 ng/ml wtIL-13
MFI: 438
M1
1 ng/ml wtIL-13
+ 1g/ml E13KR112D
MFI: 482
M1
CELL COUNT
FLUORESCENCE INTENSITY

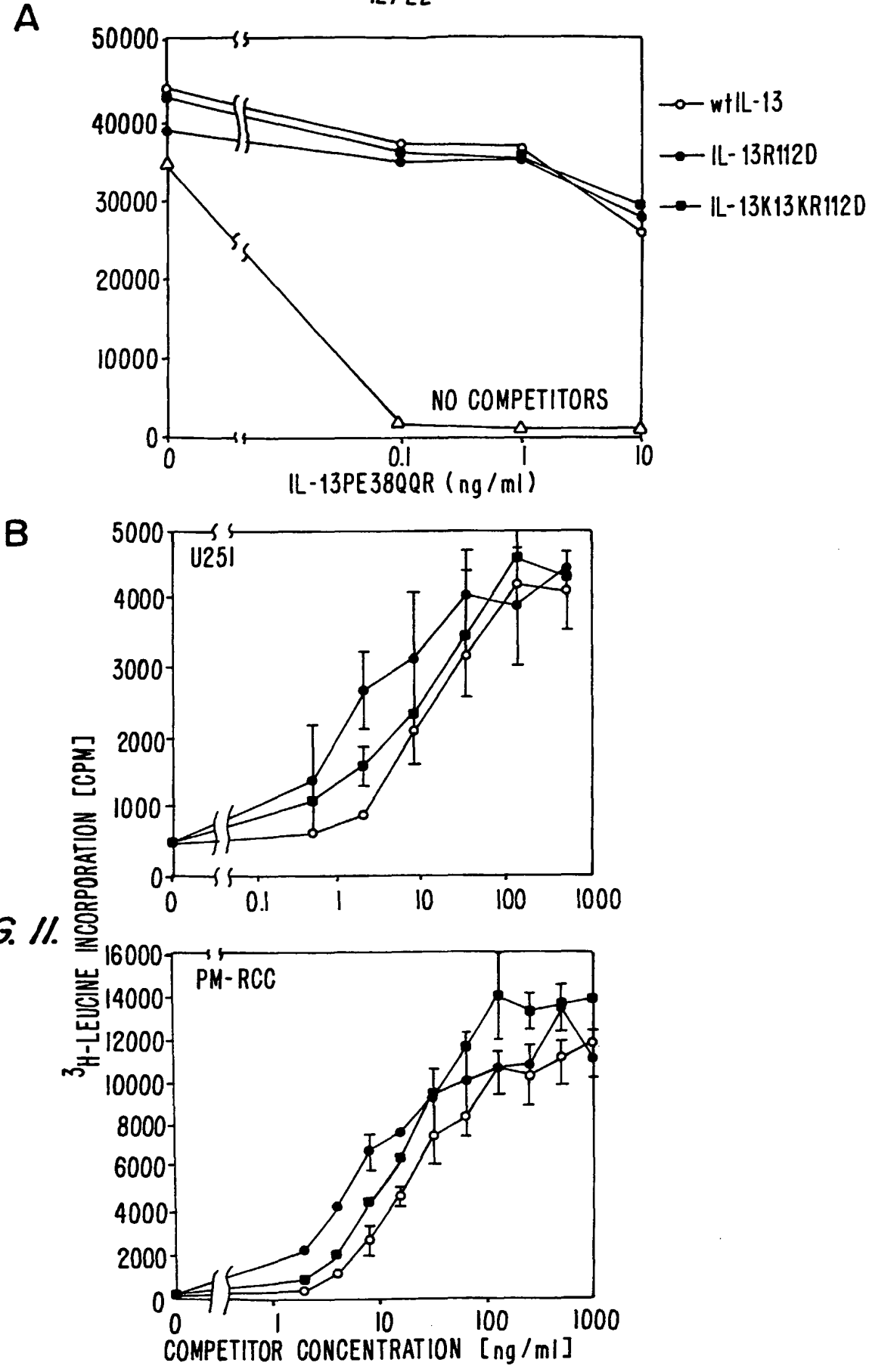

A
50000
40000
30000
20000
10000
0
0
0.1
1
10
IL-13PE38QQR (ng/ml)
NO COMPETITORS
wtIL-13
IL-13R112D
IL-13K13KR112D
B
U251
PM-RCC
^{3}H-LEUCINE INCORPORATION [CPM]
5000
4000
3000
2000
1000
16000
14000
12000
10000
8000
6000
4000
2000
0
0.1
1
10
100
1000
COMPETITOR CONCENTRATION [ng/ml]

FIG. 11.

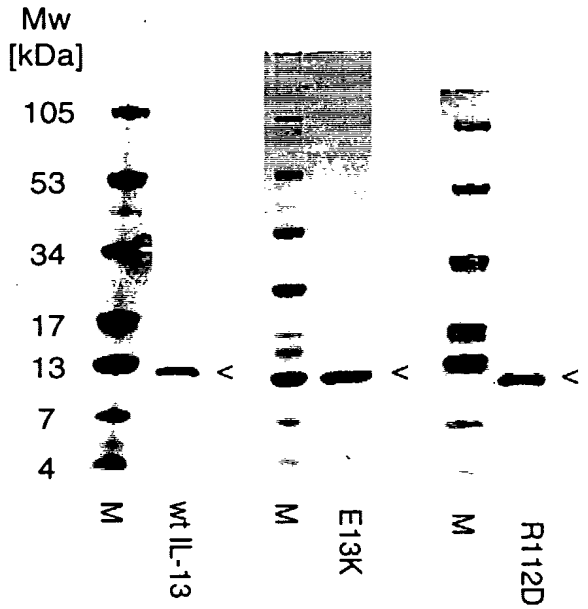


FIG. 12.

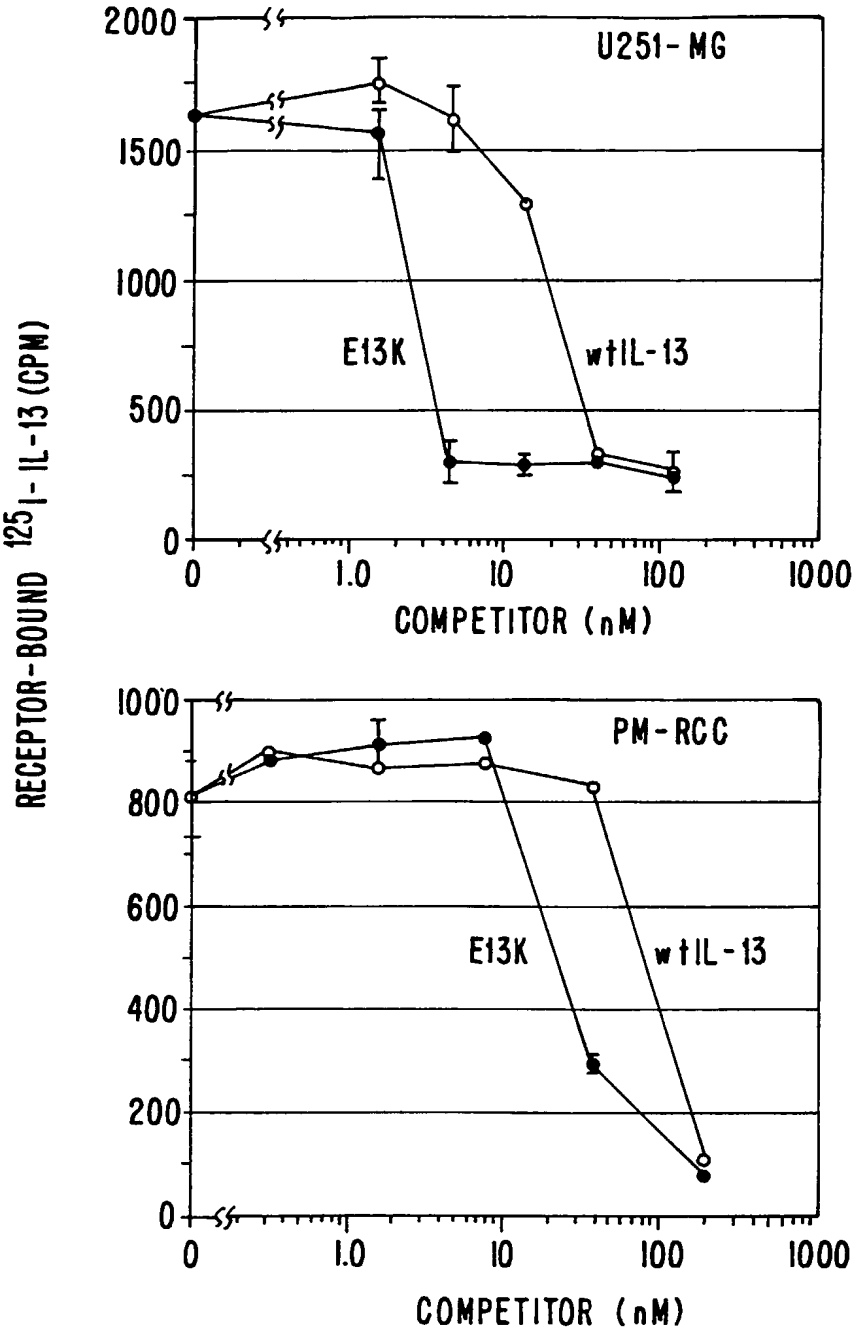

U251-MG
E13K
wtIL-13
2000
1500
1000
500
0
0
1.0
10
100
1000
COMPETITOR (nM)
PM-RCC
E13K
wtIL-13
1000
800
600
400
200
0
0
1.0
10
100
1000
COMPETITOR (nM)
RECEPTOR-BOUND ^{125}I-IL-13 (CPM)

FIG. 13.

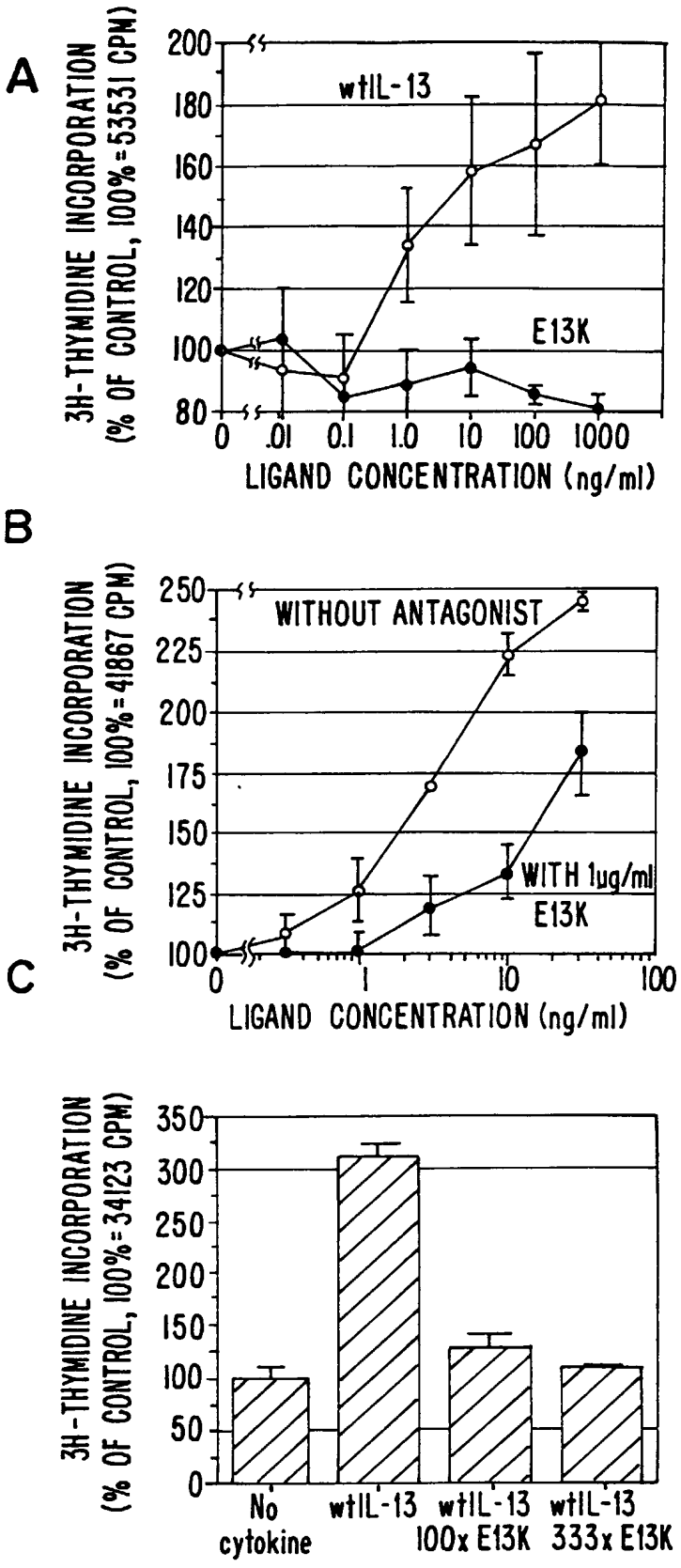

A
3H-THYMIDINE INCORPORATION (% OF CONTROL, 100% = 53531 CPM)
200
180
160
140
120
100
80
wtIL-13
E13K
0
.01
0.1
1.0
10
100
1000
LIGAND CONCENTRATION (ng/ml)
B
3H-THYMIDINE INCORPORATION (% OF CONTROL, 100% = 41867 CPM)
250
225
200
175
150
125
100
WITHOUT ANTAGONIST
WITH 1ug/ml E13K
0
1
10
100
LIGAND CONCENTRATION (ng/ml)
C
3H-THYMIDINE INCORPORATION (% OF CONTROL, 100% = 34123 CPM)
350
300
250
200
150
100
50
0
No cytokine
wtIL-13
wtIL-13 100x E13K
wtIL-13 333x E13K

FIG. 14.

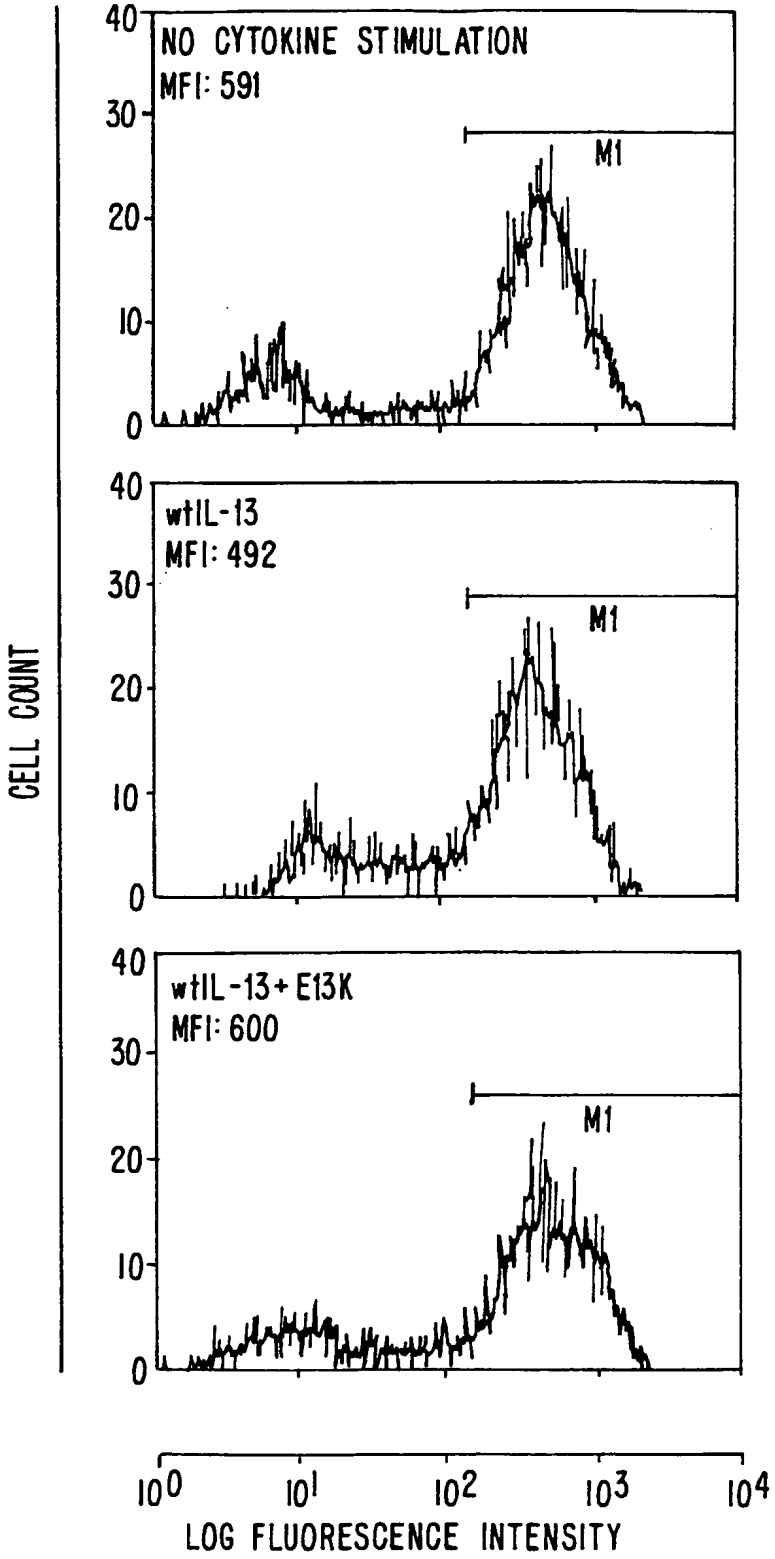

NO CYTOKINE STIMULATION
MFI: 591
M1
wtIL-13
MFI: 492
M1
wtIL-13+E13K
MFI: 600
M1
CELL COUNT
0
10
20
30
40
10^0
10^1
10^2
10^3
10^4
LOG FLUORESCENCE INTENSITY

FIG. 15.

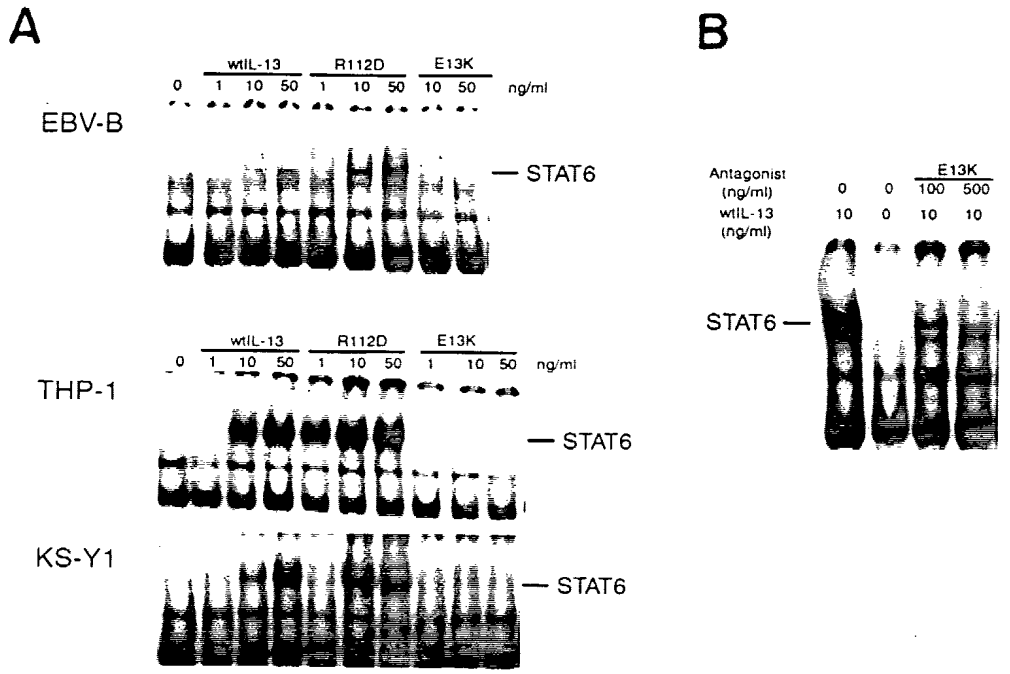

A
wtIL-13
R112D
E13K
0 1 10 50 1 10 50 10 50 ng/ml
EBV-B
STAT6
wtIL-13
R112D
E13K
0 1 10 50 1 10 50 1 10 50 ng/ml
THP-1
STAT6
KS-Y1
STAT6
B
Antagonist (ng/ml)
0 0 100 500
wtIL-13 (ng/ml)
10 0 10 10
E13K
STAT6

FIG. 16.

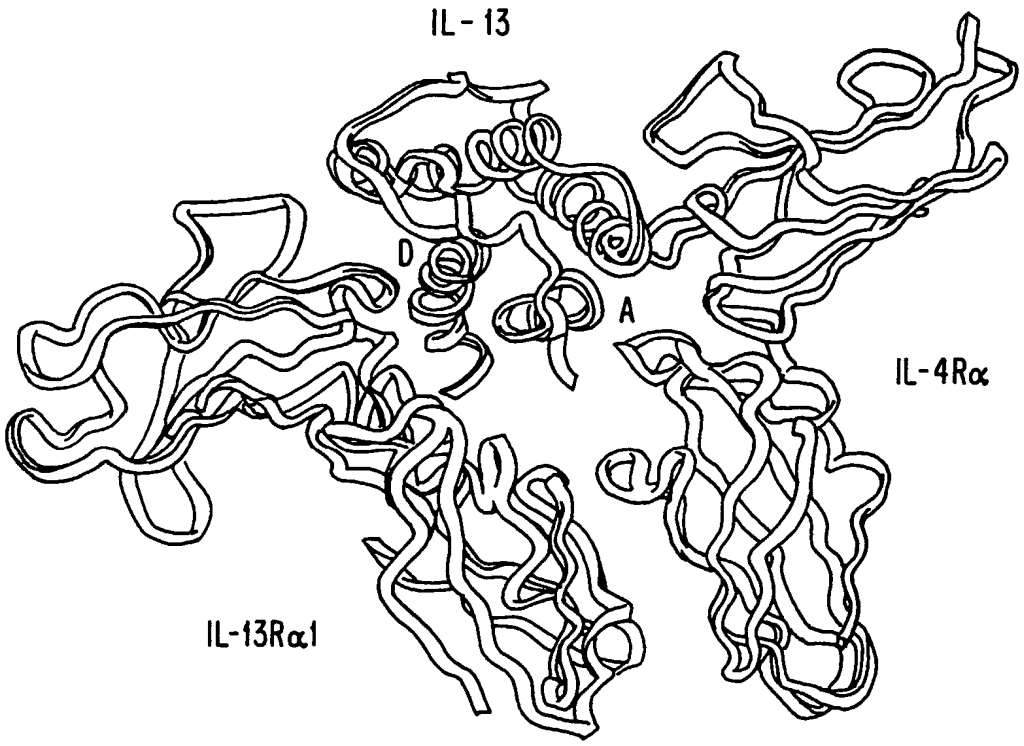
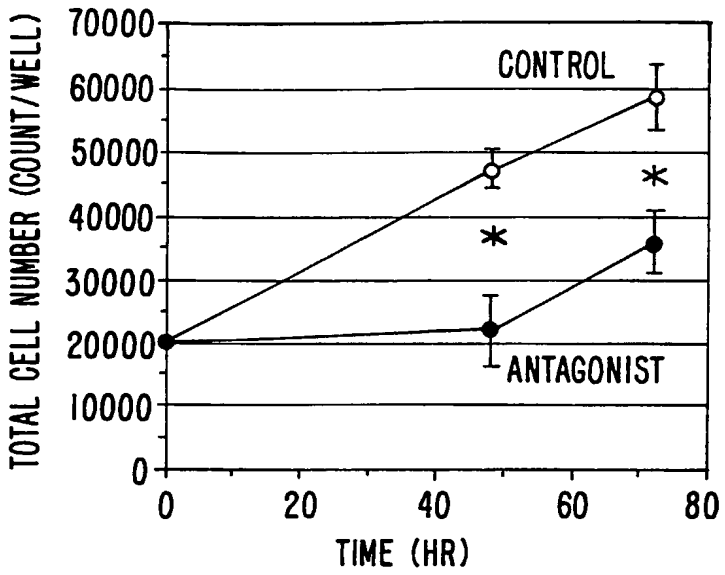


FIG. 17.

A


TOTAL CELL NUMBER (COUNT/WELL)
70000
60000
50000
40000
30000
20000
10000
0
CONTROL
ANTAGONIST
*
*
0
20
40
60
80
TIME (HR)

B

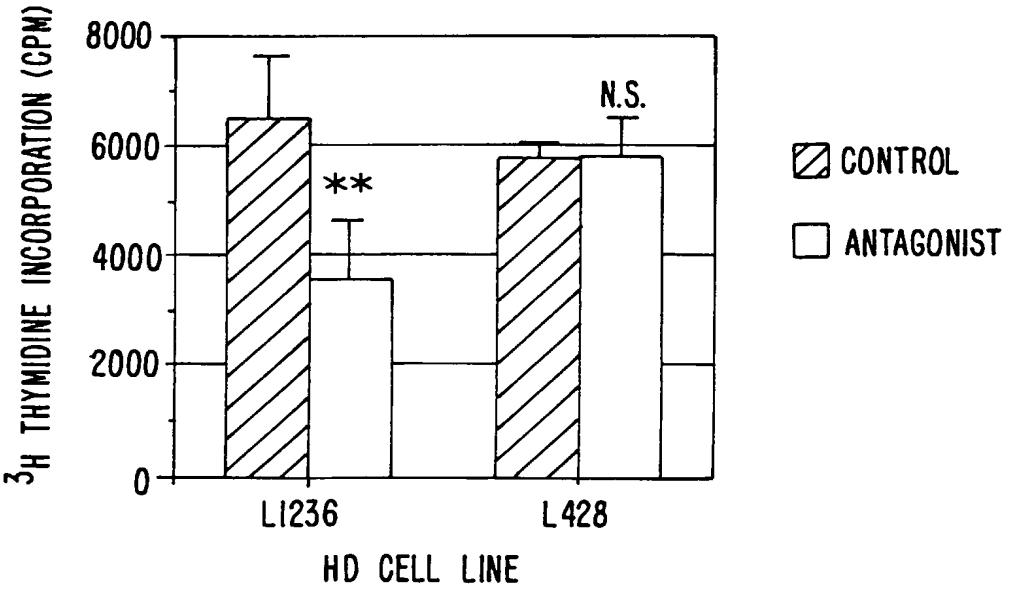
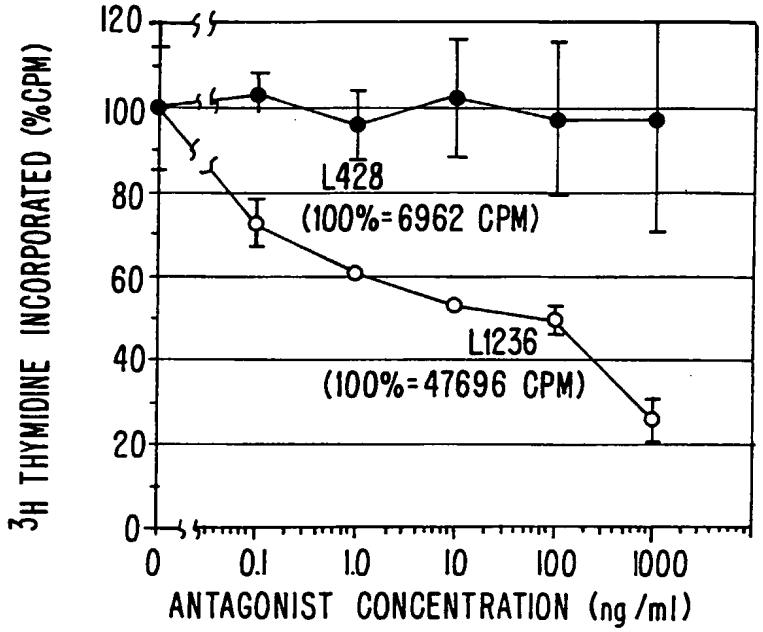

^{3}H THYMIDINE INCORPORATION (CPM)
8000
6000
4000
2000
0
**
N.S.
L1236
L428
HD CELL LINE
CONTROL
ANTAGONIST

FIG. 18.

C


120
100
80
60
40
20
0
^{3}H THYMIDINE INCORPORATED (%CPM)
L428
(100%=6962 CPM)
L1236
(100%=47696 CPM)
0
0.1
1.0
10
100
1000
ANTAGONIST CONCENTRATION (ng/ml)

D

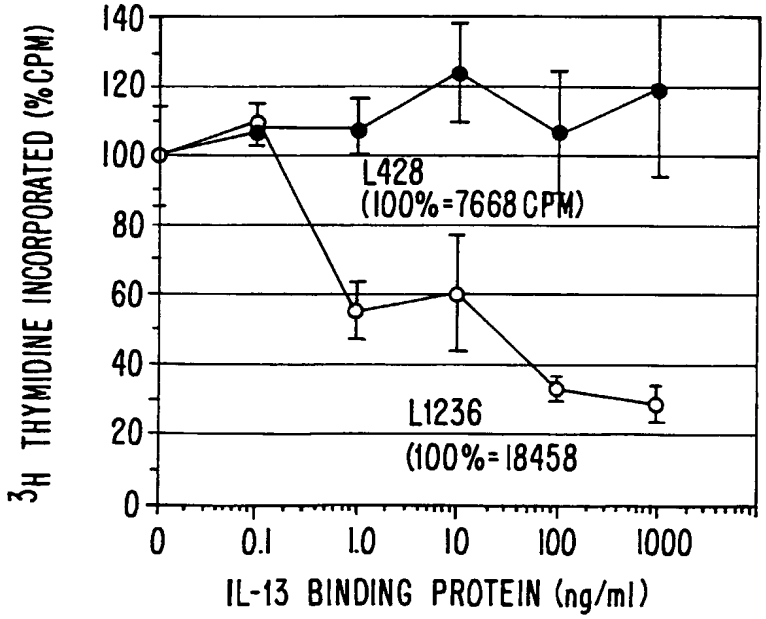
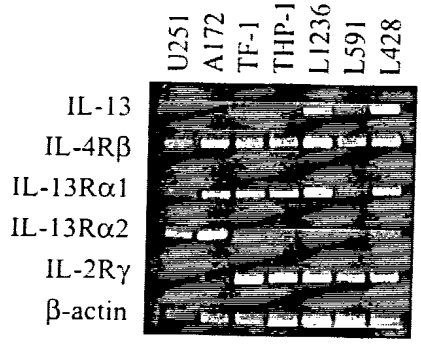

140
120
100
80
60
40
20
0
^{3}H THYMIDINE INCORPORATED (%CPM)
L428
(100%=7668 CPM)
L1236
(100%=18458
0
0.1
1.0
10
100
1000
IL-13 BINDING PROTEIN (ng/ml)

FIG. 18.

A


U251
A172
TF-1
THP-1
L1236
L591
L428
IL-13
IL-4Rβ
IL-13Rα1
IL-13Rα2
IL-2Rγ
β-actin

B

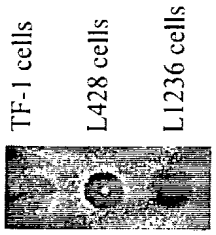
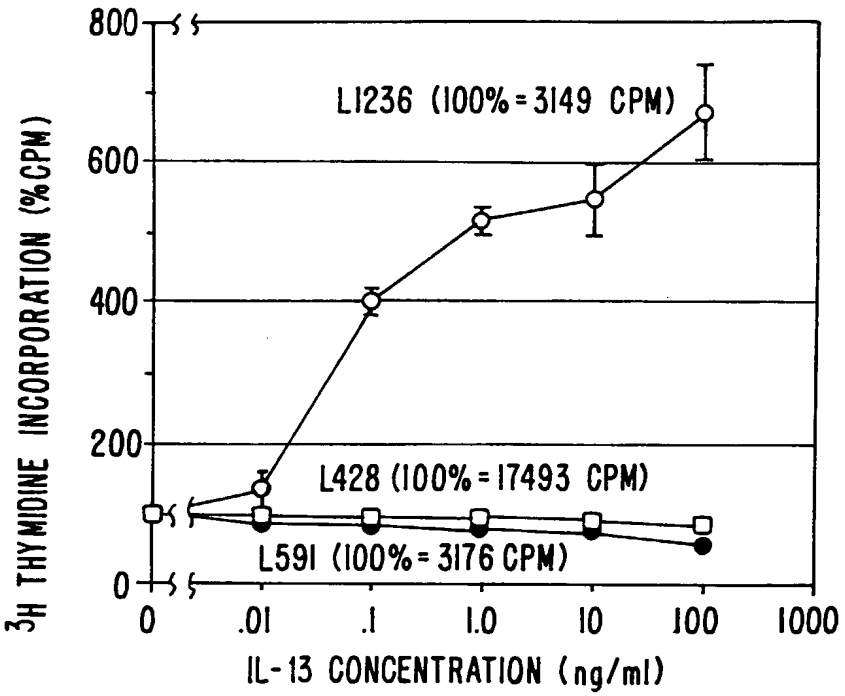

TF-1 cells
L428 cells
L1236 cells

FIG. 19.

C


800
600
400
200
0
L1236 (100% = 3149 CPM)
L428 (100% = 17493 CPM)
L591 (100% = 3176 CPM)
^{3}H THYMIDINE INCORPORATION (%CPM)
0
.01
.1
1.0
10
100
1000
IL-13 CONCENTRATION (ng/ml)

D

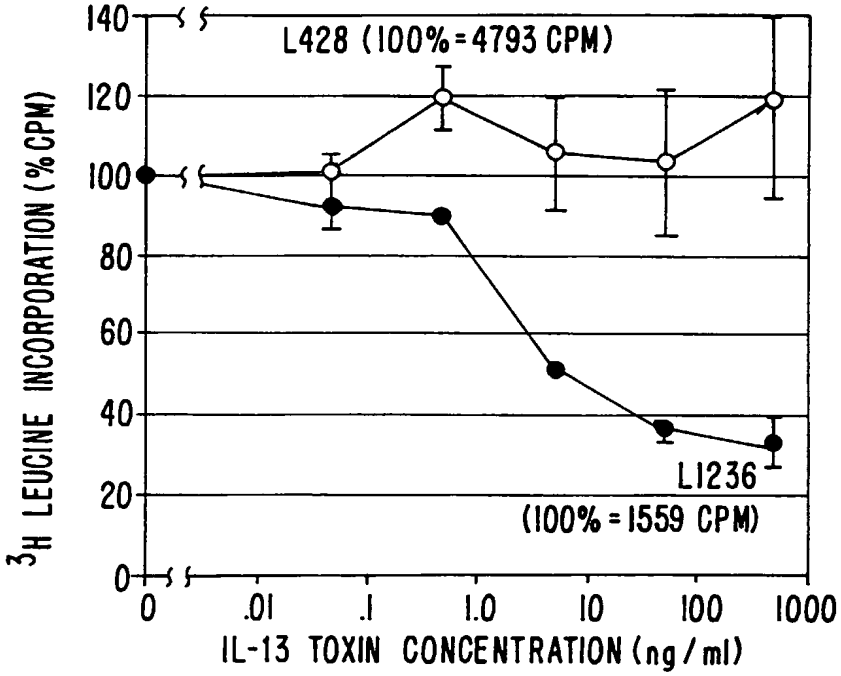

140
120
100
80
60
40
20
0
L428 (100% = 4793 CPM)
L1236
(100% = 1559 CPM)
^{3}H LEUCINE INCORPORATION (%CPM)
0
.01
.1
1.0
10
100
1000
IL-13 TOXIN CONCENTRATION (ng/ml)

FIG. 19.

amino acid. In preferred embodiments, the IL-13R binding molecules are an IL-13 or cpIL-13 with one or both of the mutations noted.

The IL-13R binding molecules of the invention have markedly higher biological activity than does wild type (wt) IL-13. In preferred forms, the molecules have at least about twice that of wtIL-13, and in even more preferred forms will have activity at least about 5 times that of wtIL-13. In the most preferred forms, the molecules have about 10 times the activity of wtIL-13. In a preferred assay, the biological activity is measured by conducting a proliferation assay in TF-1 cells.

The invention further provides chimeric molecules in which the IL-13R binding molecules described above serve as a targeting moiety. In particular, the invention provides chimeric molecules comprising which IL-13R binding molecules and one or more effector molecules. The IL-13R binding molecule can be an IL-13 or cpIL-13 comprising one or both mutations selected from the group consisting of changing an amino acid corresponding to a glutamic acid at position 110 of IL-13 to a positively charged amino acid and changing an amino acid corresponding to a glutamic acid at position 92 of IL-13 to a positively charged amino acid. The cpIL-13 can be a cpIL-13 in which the native IL-13 is opened between residues 43 and 44 (Gly and Met respectively) to produce a cpIL-13 having Met44 as the amino terminus and Gly43 as its carboxyl terminus that specifically binds an IL-13 receptor.

The effector molecule can be a cytotoxin, a label, a radionuclide, a drug, a liposome, a ligand, or an antibody. The cytotoxin can be a *Pseudomonas* exotoxin, a *Diphtheria* toxin, ricin, saponin, gelonin, abrin, or ribosome inactivating protein. In preferred embodiments, the cytotoxin is a *Pseudomonas* exotoxin (PE) or a *Diphtheria* toxin (DT) which has been modified to reduce or eliminate its binding capacity. In particularly preferred forms, the PE is PE38, PE38QQR, PE38KDEL, and PE4E. The chimeric molecules can be a single chain fusion protein.

In a preferred embodiment, this invention provides for improved methods for specifically delivering an effector molecule to a tumor cell bearing an IL-13 receptor. The method involves providing a chimeric molecule comprising an effector molecule attached to an IL-13R binding molecule of the invention, and contacting the tumor with the chimeric molecule resulting in binding of the chimeric molecule to the tumor cell.

In another embodiment, this invention provides a method for impairing the growth of tumor cells, more preferably solid tumor cells, bearing an IL-13 receptor. In preferred forms, the tumor cell is selected from the group consisting of a renal cell